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Functional Characterization of a Bidirectional Mammalian Promoter of Two Genes for the Mitochondrial Translational Apparatus

ACADEMIC DISSERTATION
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To my parents

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LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on following original scientific communications, which are referred to in the text by their Roman numerals I-III.

I **Zanotto E**, Shah ZH, Jacobs HT (2007): The bidirectional promoter of two genes for the mitochondrial translational apparatus in mouse is regulated by an array of CCAAT boxes interacting with the transcription factor NF-Y. Nucleic Acids Res 35:664-677.

II **Zanotto E**, Lehtonen V, Jacobs HT (2008): Modulation of Mrps12/Sarsm promoter activity in response to mitochondrial stress. Biochim Biophys Acta-MCR 1783:2352-2362.

III **Zanotto E**, Häkkinen A, Teku G, Shen B, Ribeiro AS, Jacobs HT: NF-Y influences directionality of transcription from the bidirectional Mrps12/Sarsm promoter in both mouse and human cells. Biochim Biophys Acta-GRM in press.

Contribution of the author

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Ernesto Zanotto's contribution

Article 1: E.Z. performed all the experiments, optimized almost all the techniques, co-designed and actively developed the project semi-independently; co-analyzed the results.

Article 2: E.Z. designed the experiments, co-analyzed the data, performed all the experiments (except the creation of mutated or deleted human promoter constructs and their luciferase assay testing); co-wrote the manuscript.

Article 3: E.Z designed the project, performed all the laboratory work and co-analyzed the results, co-wrote the manuscript.

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chloro phenyl hydrazone
cDNA	complementary DNA
COX	cytochrome c oxidase
Cyt b	cytochrome b
D-loop	displacement loop
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FCCP	p-trifluoromethoxy carbonyl cyanide phenyl hydrazone
FMN	flavin mononucleotide
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HSP	heavy-strand promoter
H-strand	heavy-strand
MnSOD	manganese superoxide dismutase
mRNA	messenger RNA
Mrps12/RPMS12	mitochondrial ribosomal protein s12
mt	mitochondrial
mtDNA	mitochondrial DNA
NAD/H	nicotinamide adenine dinucleotide (oxidized/reduced form)
NCR	non coding region
nDNA	nuclear DNA
NRF-1	nuclear respiratory factory 1
NRF-2	nuclear respiratory factory 2
NRTIs	nucleoside reverse transcriptase inhibitors
nt	nucleotide
O_H	heavy-strand replication origin
O_L	light-strand replication origin
ORF	open reading frame
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PolG	DNA polymerase gamma
PQC	mitochondrial protein quality control

qPCR	quantitative PCR
redox	reduction-oxidation
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcriptase
Sarsm/Sars2	Seryl tRNA synthetase
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
TBE	Tris-borate buffer
TCA	tricarboxylic acid cycle
TFAM	mitochondrial transcription factor A
tRNA	transfer RNA
Twinkle	T7 gp4-like protein with intramitochondrial nucleoid localization

ABSTRACT

Despite having its own DNA, the mitochondrion imports the majority of its proteins from the nucleus which, thus, controls the organellar activities, and in a reciprocal manner mitochondria send signals to the nucleus resulting in a coordination of both genomes. Although mitochondrial function depends on the nuclear genome, the organelle still plays a pivotal role in energy production, cell proliferation, maintenance and death. The functional co-existence of proteins encoded by the two separate genomes requires an efficient coordination of gene expression ensuring correct mitochondrial function upon cell specific needs. The mitochondria-nucleus networking includes interconnection between anterograde (nucleus to mitochondria) and retrograde (mitochondria to nucleus) signals. The anterograde system consists of signals generated from the nuclear genome, in response to endogenous and exogenous stimuli, and transmitted to mitochondria. The retrograde system links mitochondrially originated stimuli to co-regulation of nuclear gene expression. Nevertheless, anterograde and retrograde signalling are still poorly understood in mammals, if better characterized in yeast. The expression of most of the eukaryotic nuclear genes is controlled at the level of transcription through *trans*-acting regulatory proteins (transcription factors) which can recognize and bind to short specific nucleotide sequences (*cis*-acting binding sites) within a promoter, and consequently can modulate (activate or suppress) the expression of genes. Despite bidirectional promoters controlling the transcription of around 11% of mammalian genes, so far only few of them have been studied and properly characterized. I addressed my research study to mapping the bidirectional promoter for the *Mrps12/Sarsm* genes. *Mrps12* (mitochondrial ribosomal protein s12) and *Sarsm* (seryl tRNA synthetase) are nuclear genes encoding components of the mitochondrial translational apparatus. In my study, I identified an array of four CCAAT boxes, all with the same orientation which, interacting with NF-Y, regulate the human and mouse *Mrps12/Sarsm* bidirectional promoter activities. The NF-Y involvement in *Mrps12/Sarsm* promoter transcriptional regulation was confirmed using a dual luciferase reporter vector in combination with EMSA and Chip. The NF-Y yeast counterpart, Hap2/3/5, was previously shown to be the main activator of transcription of nuclear genes involved in mitochondrial biogenesis and OXPHOS. Also in humans, NF-

Y was demonstrated to be important in regulating mitochondrial function, as shown elsewhere by the rescue of the pathological cytochrome c oxidase assembly mutant in the *SURF1* gene in human fibroblasts, obtained by overexpressing NF-Y subunits. Due to the particular bidirectional property of NF-Y to recognize its core binding site sequence in either orientation, i.e. as CCAAT or as ATTGG on the coding strand, I hypothesize that it could be a suitable factor to govern bidirectional promoters of this class. Inverting the core binding site from CCAAT to ATTGG, I demonstrated that the CCAAT box orientation of the *Mrps12/Sarsm* promoter is not important since, the overall promoter activity changed only minimally and transcriptional directionality was maintained. The CCAAT core binding site can be recognized by several multiprotein complex factors; including NF-Y and members of the C/EBP transcription factor family (CCAAT/Enhancer Binding Protein). I demonstrated with EMSA, that C/EBPβ could also interact with the CCAAT boxes of the *Mrps12/Sarsm* promoter. Bioinformatic analyses of the CCAAT box, within the mouse or human genomes, revealed a preferential distribution in favour of bidirectional promoters over unidirectional for both NF-Y type CCAAT boxes or in combination with C/EBP-type CCAAT boxes, especially in those promoters presenting multiple CCAAT box sites. In mammals, the nuclear respiratory factors, NRF-1 and NRF-2, were previously shown to have a key role in the regulation of nuclear genes encoding components of the mitochondrial oxidative phosphorylation (OXPHOS) system, thus linking mitochondrial function to the cell's energy demand. However, mutations affecting the putative binding site for NRF-2 in the *Mrps12/Sarsm* promoter produced only a small change in the transcriptional activity in both mouse and human. To study how the bidirectional promoter for *Mrps12/Sarsm* genes can be affected by mitochondrial dysfunction, I established a cell culture model of mitochondrial stress. Several types of human and mouse cells were treated with toxins which differently affect mitochondrial function, such as inhibitors of mitochondrial protein synthesis, and agents that bring about uncoupling or respiratory chain inhibition. Mitochondrial stress produced multiple effects on promoter activity in the different cell lines I tested and at different times of exposure. High drug doses and/or a prolonged drug exposure generally suppressed transcriptional activity in the tested cell-types: mouse NIH 3T3 or C2C12 myoblasts, human HEK293 cells or U2OS or Hela. However, a shorter drug treatment stimulated the promoter activity in mouse 3T3 or human Hela cells. ROS seemed to be part of the signaling, since the potency of different drugs in producing the transcriptional response was well correlated with the amount of ROS production. HEK 293 and 3T3 cells gave a similar increase in ROS for the same mitochondrial stress induction although exhibiting opposite promoter modulation, which was stimulated in 3T3 cells or suppressed in HEK 293. The array of the four CCAAT boxes was not directly involved on *Mrps12/Sarsm* promoter stimulation under OXPHOS stress. However, transcriptional downregulation under prolonged mitochondrial stress was CCAAT box-dependent. In conclusion: the complex mechanism of gene expression modulation-co-regulation in the nuclei and the reciprocal tight communication mitochondria and the nucleus are vital for cell biogenesis, proliferation, death and adaptation to endogenous and exogenous stimuli. A clear understanding of the mechanisms by which nucleus-mitochondrial signalling occurs, and the pathways by which perturbations are signaled, may allow knowledge at the molecular level of human diseases and thus permit a specific therapeutic intervention.

1. INTRODUCTION

Eukaryotic cell development, survival and function are controlled by two compartmented genomes, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), whose activities must be under tight but flexible co-ordination. The mitochondrion possesses its own DNA, encoding only a few but essential proteins, some subunits of the respiratory chain, and encoding the full set of mitochondrial tRNAs and two rRNAs. Thus, the larger set of mitochondrial proteins are encoded by the nuclear genome and then imported into the organelle. Mutations in mtDNA or in nDNA genes encoding mitochondrial proteins can generate mitochondrial dysfunction.

Mitochondria are not only the power plants of the cell, due to the synthesis of ATP, but are also responsible for crucial cellular processes such as calcium homeostasis, the supply of carbon skeletons for gluconeogenesis, glycolysis, biosynthesis of urea and of pyrimidine nucleosides. All together, these functions make the mitochondria essential in the eukaryotic cell-cycle, biogenesis and cell death.

Although the nuclear genome encodes most of the organellar proteins, nuclear gene expression can be modulated by signals from mitochondria, via so-called retrograde communication, which has been well characterized in yeast. However, despite the many conserved general similarities between fungal and mammalian mitochondria, human retrograde signalling still remains poorly understood (reviewed in Liu Z & Butow RA, 2006). The loss of reciprocal nucleus-mitochondrial communication, which results in a dissociation between mitochondrial functions and cellular needs, can cause a large variety of degenerative diseases (reviewed in Hüttemann M, et al. 2007). Alterations in the copy number or expression of mtDNA can be the consequence of mutated nuclear proteins that disrupt intergenomic communication.

The orchestration of gene expression at the transcriptional level is achieved by transcription factors, whose action has to be temporarily regulated by the cell's needs. Some transcription factors, such as NRF-1 and -2 seem to link the expression of the nuclear genome to the mitochondrial genome, depending on cell energetic demand (reviewed in Scarpulla R.C, 2008). The CCAAT box is one of the most ubiquitously found transcription factor binding sites in eukaryotic genomes and it mainly interacts with the transcription factor NF-Y, which can boost, suppress or regulate transcription. Loss of function for a transcription factor has severe consequences for the cell, and, as reported in the literature, even a single point mutation within a promoter can drastically change the expression of a gene, resulting in aberrant pathological manifestations (Fonseca C, et al. 2007).

During basal cellular metabolism, reactive oxygen species (ROS) are produced, especially by mitochondria. The exact function of ROS still is not clear. However there is increasing evidences suggesting that they play an important role in redox cell signalling, although abnormally high ROS concentrations can also cause cell tumorigenesis or cell death (reviewed in Storz P, 2006). The knowledge of the component pathways and their co-interaction in nucleus-mitochondria signalling could have application in therapy, including the control of the apoptotic pathway in cancer, and the possibility to delay ageing related loss of mitochondrial function.

2. REVIEW OF LITERATURE

2.1 Mitochondria

The eukaryotic cell cycle is driven by two compartmented genomes: nuclear DNA and mitochondrial DNA, whose gene expression is in continuous communication. Mitochondria are organelles localized in the cytoplasm of eukaryotic cells, and possess their own circular DNA molecule. Mitochondria have an important role in cells, being the so-called "power plant" responsible for energy production, in the chemical form of ATP. Mitochondria have also other important roles, in calcium and iron homeostasis, amino acid metabolism, biosynthesis of FeS clusters and heme and in the co-regulation of apoptosis (reviewed in Garesse R & Vallejo CG, 2001).

2.1.1 Evolution and origin of mitochondria

The mitochondrial presence in the cell is thought to be the consequence of an evolutionary intracellular endosymbiotic process which originated via the invasion of a protoeukaryote by bacteria (reviewed in Taylor FJ, 1979; Poole AM, 2007). This has resulted in two distinct active genomes within eukaryotic cells. However, during evolution the mitochondrial genome was reduced in size, following organelle DNA transfer to the nucleus, giving rise to mitochondrial genes actively expressed within the nuclear genome. Endosymbiotic gene transfer is thought to be part of a beneficial evolutionary process which, therefore, resulted in a reduced mtDNA capacity to encode only a few proteins, whereas most of the proteins found in mitochondria are encoded by the nuclear genome and then imported into mitochondria (reviewed in Timmis JN, et al.2004).

Mitochondrial ROS production can cause accumulation of damaged mtDNA, mutated or deleted, which will result in mitochondrial dysfunctions with deleterious effects for the affected cells and organisms (see paragraph 2.5.3). Thus evolution was probably in favour of those organisms, in which part of the mitochondrial genome could migrate and integrate within the nuclear genome which is, under normal conditions, less exposed to ROS than the mitochondrial genome (reviewed in Saccone C, et al. 2000).

Perhaps, a such evolutionary process was more feasible for some mitochondrial genes than others, considering also the high selective permeability of the mitochondrial inner membrane. Non less, the nuclear localization of a gene would represent an advantage over the mitochondrial localization during sexual reproduction. In sexual reproduction, the offspring receives two alleles of a nuclear gene, one from the male and the other from the female, rather than the set of genes from only the female as it occurs for mitochondrial DNA transmission (reviewed in Saccone C, et al. 2000).
Furthermore, the nuclear localization of genes encoding mitochondrial proteins can enforce the nuclear control on mitochondrial functions, which are modulated upon cell's needs.

2.1.2 Structure of mitochondria

Mitochondria are compartmented by two membranes, the outer and inner membranes, which delimit an intermembrane space and the internal matrix. Mitochondria are distributed within the cells to form a dynamic and plastic reticulum (Aon MA, et al. 2006). Their number per cell and shape are very diverse and present tissue- specific characteristics, specially reflecting the energetic demand level of the cell. However, drastic changes can always occur during development or toxic conditions, such as mitochondrial swelling induced by non-steroidal anti-inflammatory drugs (NSAIDs) or by an exposure to toxic amounts of Ca^{2+} ionophores (reviewed in Bereiter-Hahn J & Voth M, 1994; O'Connor N, et al. 2003). Upon swelling, mitochondria change from a rod to a larger spherical shape, characterized by loss of the outer membrane integrity, which results in collapse of the membrane potential and loss of mitochondrial function (reviewed in Kaasik A, et al. 2007). Mitochondria undergo dynamic remodeling processes like fission and fusion, which are driven by nuclear encoded proteins such as Fzo1p, Mfn1, Mfn2, OPA1 and Mgm1p (reviewed in Chen H & Chan DC, 2005). During such dynamic processes mitochondria might also exchange genomes between them (Nakada K, et al. 2001). Mitochondria do not have a random distribution within the cell but instead they normally interact with the cytoskeleton which can drive their localization and movements. Mitochondrial localization is influenced by retrograde and anterograde stimuli. Nitric oxide, local ADP concentration, ageing, synaptic activity and calcium level have been reported as some of the factors influencing neuronal movements of mitochondria (reviewed in Frederick RL & Shaw JM, 2007).

The outer membrane, rich in pores, is permeable also to many peptides up to 5000 Da in size, whereas the inner membrane permeability is very strictly selective and mediated by specific transporters (reviewed in Turcotte LP, 2003). The inner membrane is folded into cristae which vastly increases its surface area.

The inner membrane can be dissected into two parts: The inner boundary membrane is located close to the outer membrane. The two membranes are in contact, transiently fused in many places, creating channels for protein import or other exchange functions (Schülke N, et al. 1997). The second part is called the cristae, lamellar structures in the matrix, which are connected to the inner boundary membrane through small tubular structures called cristae junctions. The cristae have particular importance being the surface in which the OXPHOS complexes are located (Perkins G, et al. 1997). Evidence suggests, that the number and morphology of the cristae is related to the mitochondrial response concerning the energy demand of the cell (reviewed in Scheffler IE, 1999). Tissues with a high respiratory rate, such as muscles and neurons, present highly folded, lamellar cristae with a large surface area (reviewed in Scheffler IE, 1999).

2.1.3 Membrane transporters

The endosymbiotic generation of compartmented organelles within the cell required the evolution of a protein import machinery, facilitating the internalization of nuclear gene encoded proteins into the mitochondria (reviewed in Dolezal P, et al 2006). Most of the proteins to be imported present a targeting signal that ensures their delivery into the mitochondria. The targeting signals can be internal within the protein or cleavable leader presequences. The leader presequence consists typically of a 20-30 amino acid cleavable peptide located at the N-terminal domain of the protein to be imported, and it is recognized by the protein import machinery (reviewed in Kutik S, et al. 2007).

The TIM and TOM complexes are the main protein translocase components of the protein import machinery. The TOM complex (translocase of the outer membrane of mitochondria) is responsible for translocating proteins across the mitochondrial outer membrane. Among the several integral membrane protein components of the TOM complex, TOM 70 and TOM 20 are the receptor subunits which recognize and bind the substrate proteins to be imported, that can then pass through a translocation channel and reach the mitochondrial intermembrane space (reviewed in Endo T & Kohda D, 2002). The imported proteins can now interact with two distinct import machineries located within the inner membrane, the TIM 22 and TIM 23 complexes.

The TIM22 (translocase of the inner mitochondrial membrane) complex mediates the transfer of proteins that will integrate into the inner membrane, whereas the TIM23 complex forms a channel that mediates protein entry into the mitochondrial matrix (reviewed in Dolezal P, et al. 2006). The newly synthesized proteins are bound to the chaperones Hsp70 and Hsp90 which mediate protein transit through the cytoplasm, preventing protein degradation, and following the recognition by TOM 70 and TOM20 receptors, the chaperones release the protein precursor to the TOM complex (Bhangoo MK, et al. 2007). Once the protein precursors are correctly localized in the mitochondrion, the leader presequence is proteolytically cleaved off by various processing peptidases, and the protein is folded, thus activated, by molecular chaperones (reviewed in Gakh O, et al. 2002). HSP60 and HSP10 represent the main molecular chaperones, which regulate protein folding, but also exert a critical role in mitochondrial protein quality control (PQC), a mechanism responsible for ensuring the functional integrity of the proteins, protein (re) folding, protein protection against aggregation, and the specific proteolytic removal of damaged polypeptides. The PQC is important during cellular growth in normal conditions, and is essential for the cell’s survival during stress conditions, originated in the cytoplasm or in mitochondria, which increase the number of inactive, denatured, or damaged polypeptides (reviewed in Leidhold C & Voos W, 2007).

2.2 Oxidative Phosphorylation

The endosymbiotic process probably arose and proceeded during evolution due to the ability of some bacteria to use molecular oxygen, as electron acceptor during the aerobic redox reaction for energy production, in the form of ATP. ATP production within a cell occurs through the chemiosmotic process (Mitchell P, 1961). The electron transport chain, located in the inner membrane, produces an electrochemical proton gradient, with a high accumulation of protons in the intermembrane space compared to the matrix. The energy required to pump and accumulate protons is derived from the redox proton translocation across the electron transport chain, which ends with the reduction of the molecular oxygen to H_2O by respiratory chain complex IV (Figure 2.1. Mitochondrial OXPHOS). The energy accumulated from the proton gradient is then used to synthesize ATP from Pi (inorganic phosphate) and ADP by the ATP synthase, while protons flow down the gradient to drive the reaction (reviewed in Matsuno-Yagi A & Hatefi Y, 1988).

2.2.1 The electron transport chain

The electron transport chain is made up by four multisubunit enzymatic complexes, I, II, III and IV whose subunits are encoded by both the mitochondrial and nuclear genomes.

Complex I (NADH:ubiquinone oxidoreductase): is the largest complex composed of at least 42 subunits, of which 7 are encoded by the mitochondrial DNA. It catalyses the reduction of ubiquinone (CoQ) by transferring electrons from NADH previously reduced during the metabolism of pyruvate, aminoacids and fatty acids. The redox reaction catalyzed by complex I pumps four protons from the matrix side to the mitochondrial intermembrane space (reviewed in Hirst J, 2005). Complex I does not exist in all organisms on the contrary of the other members of the electron transport chain. NADH dehydrogenase, the yeast functional counterpart of mammalian complex I, was able to correct deficiency of complex I in human and rodent cells (Seo BB, et al. 2006) Rotenone can inhibit complex I activity.

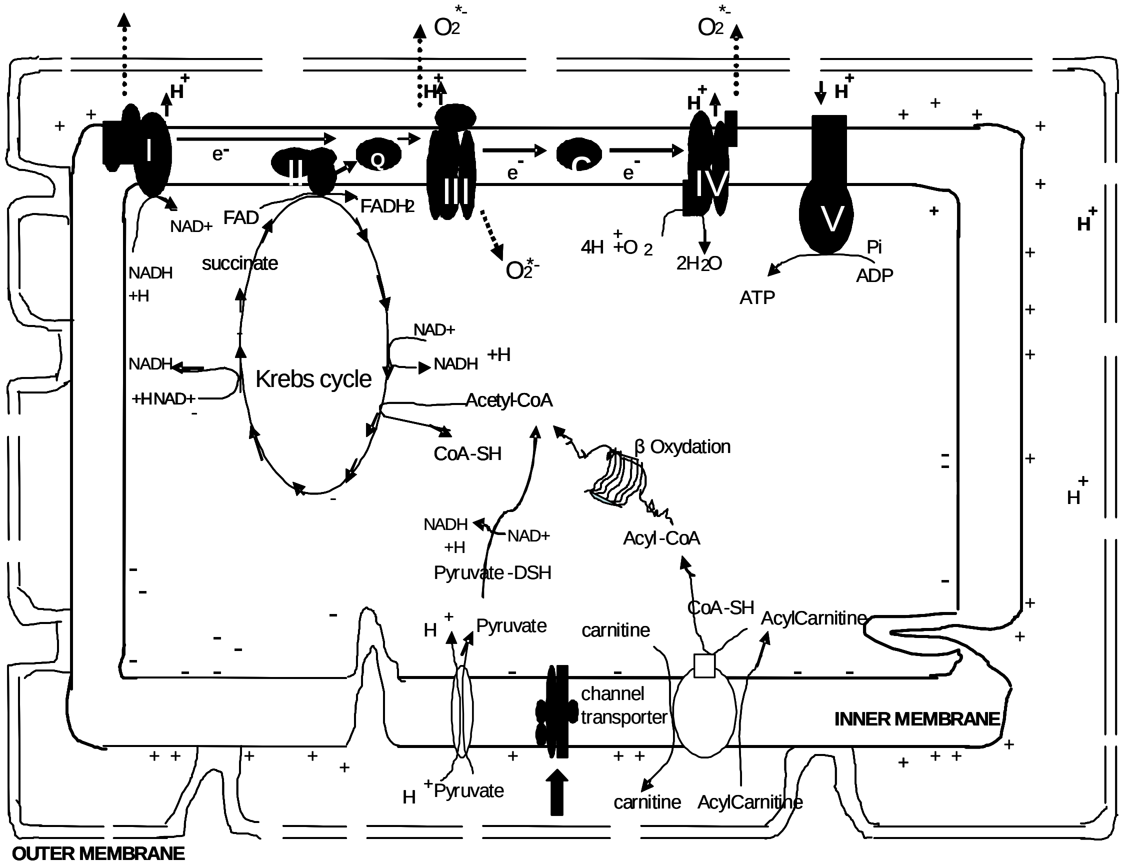


Figure 2.1.Mitochondrial OXPHOS: Electrons (e^-) are transferred from complexes I, II, III and through complex IV to oxygen, reducing it to H_2O. Ubiquinone (Q) and cytochrome c (C) are mobile electron transporters. Electron flow through complexes I-III and IV is accompanied by proton (H^+) translocation from the matrix to the intermembrane space. The resulting proton gradient is used by complex V to synthesize ATP. After internalization into mitochondria acyl-carnitine is catabolized to produce acetyl-CoA, which enters the Krebs cycle to produce NADH and $FADH_2$
(From Morin Christophe, Universite' Paris XII)

Complex II (succinate:ubiquinone oxidoreductase): is the smallest and structurally simplest complex, consisting of only four subunits, all encoded by the nuclear genome. Complex II links the Kreb cycle directly to the electron transport chain (Scheffler, 1999) by transferring to ubiquinone (CoQ) the electrons acquired from the oxidation of succinate to fumarate in the Krebs cycle. The reduced CoQ then transfers electrons to complex III (reviewed in Cecchini G, 2003). Complex II does not have proton pumping activity.

Complex III (ubiquinol-cytochrome c oxidoreductase): in mammals, the three functionally catalytic subunits are the cytochromes *b* and *c1* and the Rieske iron-sulfur protein, the ones participating in electron transfer and proton pumping. Only one subunit, cytochrome *b*, is encoded by mtDNA. Complex III catalyses the reduction of cytochrome *c* by ubiquinol, and transfers protons into the intermembrane space. The cytochrome c is located between complexes III and IV on the outer side of the inner membrane and it transfers electrons between complex III and IV (Trumpower BL, 1990). Complex III activity is antimycin sensitive.

Complex IV (cytochrome c oxidase): in mammals, is made up of 13 subunits, three of which are encoded by mtDNA. Complex IV is the terminal electron acceptor and it catalyses the reduction of molecular oxygen to water, while pumping protons into the intermembrane space (reviewed in Kadenbach B, 2000). Complex IV activity is KCN sensitive.

The flow of electrons across the first four complexes, generated by the oxidation of NADH, at the level of complex I, and $FADH_2$, at the level of complex II, produces the energy required to create an electrochemical proton gradient, which in turn is used by complex V to synthesize ATP.

Complex V (ATP synthase): The complex V is responsible for the synthesis of ATP from ADP and inorganic phosphate, via a unique rotary mechanism, using as energy source the proton gradient with which it is coupled. It can be divided into two main parts: the membrane-embedded domain Fo, through which the protons flow, and the catalytic F_1 ATPase unit that is located towards the matrix. The subunits forming Fo subcomplex are nuclearly and mitochondrially encoded. The F_1 complex consists of five nuclearly encoded subunits: α, β, γ, δ, and ε. The γ subunit rotation changes the catalytic site that can function in the synthesis or hydrolysis of ATP.

However ATP hydrolysis can be blocked under de-energized situations by MgADP that blocks rotation (reviewed in Boyer PD, 1998). ATP synthase activity can be inhibited by oligomycin.

2.3 Metabolic pathways inside mitochondria

2.3.1 The Krebs cycle

The citric acid cycle has catabolic functions, being the final common pathway for the oxidation of molecules from nutrients; it also possesses anabolic functions, since some Krebs cycle intermediates are used to form amino acids and precursors for other biosynthetic reactions e.g. the succinyl-CoA is a precursor for the heme synthesis. The majority of nutrients are catabolized initially outside the mitochondria generating acetyl-CoA, which then can enter the cycle. The acetyl-CoA is derived by the decarboxylation of pyruvate and by β-oxidation of fatty acids (reviewed in Scheffler IE, 1999). The Krebs cycle consists of a series of eight catalytic enzymes which catabolize the metabolic substrates and finally reduce NAD+ and FAD that will then serve as electron donors to the ETC (reviewed in Scheffler IE, 1999). Some of the substrates entering the Krebs cycle are succinate and acetyl-CoA which is produced by the β-oxidation or by pyruvate decarboxylation through the pyruvate dehydrogenase complex (reviewed in Hertz L, 2007). The cycle requires always the presence of oxidized NAD+ and FAD thus it can function only under aerobic conditions. The Krebs cycle speed is regulated by the ATP demand of the cells and by feedback mechanisms (reviewed in Scheffler IE, 1999).

2.3.2 Fatty Acid metabolism: beta –oxidation

Beta-oxidation is connected to the Krebs cycle via acetyl-CoA as intermediate. Fatty acids, which are stored as triacylglycerols in adipose tissues, are first hydrolyzed to form acyl-CoA. The acyl group is then transferred to carnitine to form acyl-carnitine which is then transported into the mitochondrial matrix by a specific carrier protein, carnitine-acylcarnitine translocase. Once in the matrix, carnitine is released and acyl-CoA is then re-formed. Acyl-CoA is then catabolized by a repeated series of four reactions: oxidation through FAD reduction, hydration, oxidation through NAD+ reduction and in the last reaction two carbon units are split off from the acyl-CoA to give acetyl-CoA. The acetyl-CoA can then enter the Krebs cycle (reviewed in Bartlett K & Eaton S, 2004).

2.3.3 Urea cycle

The urea cycle is an essential pathway for the conversion of the amino acid nitrogen group into its extractable form, urea. The urea production occurs in the liver, although many enzymes of the urea cycle are highly expressed also in other tissues where they synthesize nitric oxide. The urea cycle consists mainly of 5 enzymes, of which only ornithine transcarbamylase and carbamyl phosphate synthetase-I are located in the mitochondrial matrix (reviewed in Morris SM, 2002).

Nitric oxide is produced during the conversion of L-arginine to citrulline by the nitric oxide synthase. However, L-arginine can also be metabolized by the arginase enzyme to produce urea (Smith HA, et al. 2006).

In the liver, glucagon, insulin and glucocorticoids couple urea cycle activity to amino acid nitrogen flux, which depends on the dietary protein intake or on the catabolism of endogenous proteins. In other non-hepatic tissues, the expression of urea cycle enzymes is regulated by pro- and anti-inflammatory cytokines and other agents (Morris SM, 2002).

2.3.5 Coenzyme Q

Coenzyme Q is involved in many mitochondrial and cellular processes, mainly due to its oxidation/reduction property, that resides in the quinone group. As previously seen, coenzyme Q is involved in electron flow through the electron transport chain, mediating electron transport between complex I and III and other dehydrogenases. While mediating proton transfer, coenzyme Q is also transferring protons to the mitochondrial intermembrane space, thus directly contributing to the creation of the proton gradient. When coenzyme Q is reduced by complex I, it takes protons from the mitochondrial matrix, and it releases the protons outside when it is re-oxidized by complex III (reviewed in Crane FL, 2001).

Coenzyme Q is also distributed in all the membranes within the cell, contributing to broad antioxidant effects. Similarly in the membrane are located many enzymes which can reduce any coenzyme Q quinone radical generated by reaction with a lipid or oxygen radical. Some of these enzymes are NADH cytochrome b5 reductase, NADH/NADPH oxidoreductase (DT diaphorase) and NADPH coenzyme Q reductase (reviewed in Nohl H, et al. 2001). Modulating the ROS level, coenzyme Q influences the cellular redox state, which is part of intracellular signalling (see below). The cellular coenzyme Q is mainly endogenous and is synthesized in the mitochondria. However as a consequence of a decreased level of coenzyme Q during ageing, the coenzyme Q level can be increased by dietary supplementation. Several clinical trials are investigating the possible beneficial effects of coenzyme Q for the treatment of some neuro-degenerative pathologies (reviewed in Crane FL, 2001; Galpern WR & Cudkowicz ME, 2007).

2.4 The mitochondrial genome

The human mitochondrial genome consists of a 16.6 kb circular double-stranded DNA molecule lacking introns. MtDNA is composed of 2 strand types, heavy (H) and light (L). The strands differ in GT content, and thus also in buoyant density and can be separated by CsCl density gradient centrifugation. The mtDNA encodes 13 polypeptides, 2 rRNAs and 22 tRNAs. The 13 polypeptides are subunit components of four complexes in the OXPHOS system: seven are subunits of complex I (ND1, 2, 3, 4, 4L, 5 and 6), one is a subunit of complex III, cytochrome *b*, three are subunits of complex IV (COX I, II, III), and two are subunits of ATP synthase (A6, A8) (See Figure 2.2. The human mitochondrial genome). Most of the coding genes are located on the H-strand. The mtDNA appears genetically compact, since it has only a 1.1 kb noncoding region known as the D-Loop that has slightly variable sequence even within a species. Within the D-Loop lie the promoters for H- and L-strand transcription initiation. This promoter region is proposed to be the site of location of the origin of H-strand replication (O_H) as well as the major control region for mtDNA replication (reviewed in Falkenberg M, et al. 2007).

2.4.1 Nucleoid organization

The mitochondrion contains many copies of its DNA molecule which are associated with proteins in complex structures called nucleoids. In human cells there are several thousand nucleoids, depending on the cell type. Each nucleoid contains many mtDNA molecules as well as many proteins responsible for the replication and maintenance of mtDNA (reviewed in Malka F, et al. 2006). Among the proteins structuring a nucleoid we can find the mitochondrial transcription factor TFAM, the mtDNA helicase Twinkle, the mitochondrial single stranded DNA binding protein (mtSSB) and both subunits of DNA polymerase γ (POLG); plus other enzymatic proteins required in mtDNA maintenance, transcription and replication (reviewed in Holt IJ, et al. 2007). Nucleoids are highly dynamic structures, redistribute actively during mitochondrial fission and fusion and are considered to be the mitochondrial units of DNA inheritance (Wang Y & Bogenhagen DF, 2006).

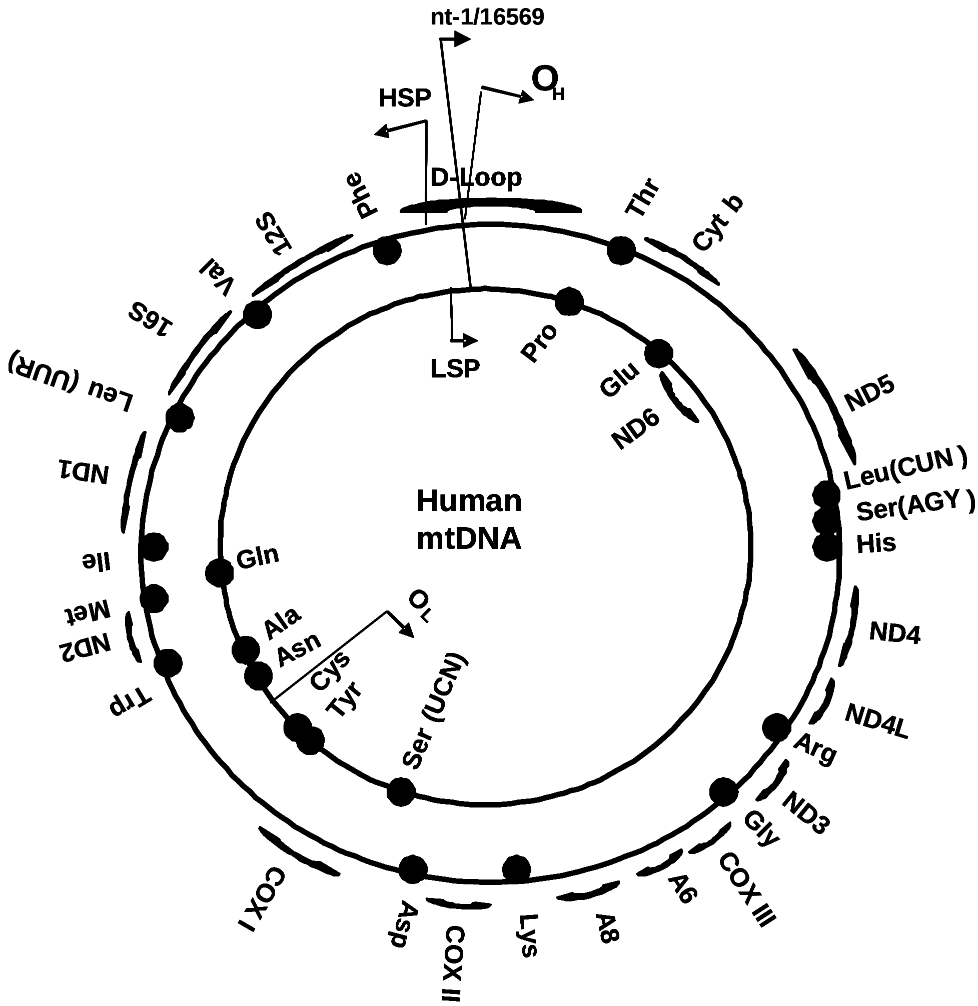


Figure 2.2. The human mitochondrial genome (not to scale), 16.6 kb. The mtDNA encodes 13 polypeptides, 2 rRNAs (12S and 16S), and 22 tRNAs. Genes encoded by the light strand are marked inside the circle, HSP and LSP represent heavy- and light-strand promoters; O_H and O_L proposed origins of heavy and light-strand replication.

The mitochondrial genome is maternally inherited. Within a mitochondrion the mtDNA molecules are considered to be all the same in sequence, representing a state denoted as homoplasmy, as seen for most individuals (Dujon B, 1981). Heteroplasmy, however, the co existence of different mtDNA sequences, wild-type and mutated mtDNA, can occur following alterations in the mtDNA sequence, such as point mutations or deletions (reviewed in Wallace DC, 1993). Mutations in mtDNA can be either inherited or spontaneous, therefore before contributing to organellar dysfunctions within a cell mutations need to occur at a enough high level, above a certain threshold, compared to the wild-type mtDNA level. Mutant mtDNA can accumulate within a cell following unequal redistribution of mtDNA in dividing cells or as consequence of a rapid self replication, causing mitochondrial dysfunction in a tissue-specific-manner (reviewed in Wallace DC, 1993). The mutation rate in mitochondrial DNA occurs 5 to 10 times faster than observed for nuclear DNA, per site, per year for the whole genome, excluding the control region (reviewed in Pakendorf B & Stoneking M, 2005).

2.4.2 Translation system

Mitochondria possess their own translational machinery for the synthesis of mtDNA-encoded proteins. MtDNA encodes some RNA components of the translational apparatus, namely two rRNAs, 12S and 16S and the full set of 22 tRNAs. The other (protein) components of the mitochondrial translational machinery are encoded by the nuclear DNA (reviewed in Clayton DA**.** 2000). Further evidence supporting the bacterial origin of mitochondria is the similar sensitivity to antibiotics between the mitochondrial and bacterial translational apparatus. Antibiotics cause comparable impairment of protein synthesis at similar concentration in the two cases. Mitochondria present a unique genetic code to translate genes, different from that of nuclear DNA (reviewed in Jukes TH & Osawa S, 1993). In human mitochondria, AUA codes for methionine instead of leucine; UGA codes for trypthophan instead of being a stop codon; AGA and AGG are the stop codons instead of coding for arginine (reviewed in Barrell BG, et al 1979). The AUG start codon is absent in some mRNAs. The protein translation starts with fMet-tRNA (reviewed in Attardi G, 1985). Many other proteins involved in the mitochondrial translational apparatus are imported from the cytosol, such as the aminoacyl-tRNA synthetases, TFB (-1M and -2M), factors for initiation, elongation and termination (Cotney J, et al. 2007; reviewed in Jacobs HT & Turnbull DM, 2005).

Ribosomes, complexes of RNAs and proteins, represent the core protein synthesis

apparatus in both cytosol and mitochondria, they translate messenger RNAs into polypeptide chains. The genetic instructions for protein synthesis are held by messenger RNAs, which bind to Ribosomes. Following mRNAs binding, tRNA-aminoacid molecules can interact with the complex Ribosome-mRNA and initiate polypeptide synthesis. tRNA, RNA transfer, is a small RNA molecule, with an active amino acid bound to its 3´ terminal site and it possesses a three base region called anticodon that can base pair to the corresponding three base codon region on mRNA. The pairing between mRNA codon and tRNA anticodon directs protein synthesis by adding a specific amino acid to the nascent polypeptide. Each type of tRNA molecule can be attached to only one type of amino acid, but tRNA molecules bearing different anticodons may also carry the same amino acid as consequence of the so called degenerate genetic code. The linkage between tRNA and the specific amino acid is catalyzed by amino acid specific aminoacyl tRNA synthetases (reviewed in Duchêne AM, et al. 2009).
Aminoacyl tRNA synthetase (aaRS) is an enzyme that catalyzes the esterification of a specific amino acid or its precursor to one of its compatible cognate tRNAs to produce an aminoacyl-tRNA. The full set of mitochondrial aminoacyl tRNA synthetases is nucleus-encoded, synthesized on cytosolic Ribosomes and imported into mitochondria. In human, nine genes coding for each mitochondrial aminoacyl tRNA synthetases have been cloned and their product characterized (IleRS, PheRS, LysRS, LeuRS, TrpRS, SerRS, MetRS, TyrRS, AspRS). Other putative mitochondrial aaRSs were identified in silico (CysRS, GluRS, ValRS, AlaRS, AsnRS, ProRS, ThrRS). Mitochondrial aaRSs are normally encoded from a single gene, however, in two cases, for GlyRS and LysRS, the same gene was shown to encode both the cytosolic and mitochondrial aaRSs. Two translation initiation sites generate the two GlyRS enzymes. While the LysRS gene encodes the two enzymes through alternative splicing. The gene encoding mitochondrial GlnRS is not yet identified and Gln-tRNA^{Gln} is probably formed by the transamidation of Glu-tRNA^{Gln} , this reaction is catalyzed by a tRNA-dependent amidotransferase (AdT). Glu-tRNA^{Gln} synthesis is catalyzed by GluRS (reviewed in Duchêne AM, et al. 2009).

Recently, TFB1M and TFB2M were shown to be important factors involved in the regulation of mitochondrial gene expression and translation in mammals and *Drosophila*

(Adán C, et al. 2008; Metodiev MD, et al. 2009). Both TFB1M and TFB2M function also as methyltransferases, they can bind to s-adenosyl methionine, which acts as methyl donor for the methyltransferase reaction (Cotney J, et al. 2007). In order to elucidate the physiological roles of the two protein factors, *Drosophila* and mouse models with deficiency for TFB1M or TFB2M have been created by some groups.

TFB2M knockdown by RNA interference in *Drosophila melanogaster* caused impairment of mtDNA transcription resulting in strong decrease of oxidative phosphorylation and mitochondrial ATP synthesis and caused lethality by arresting development at larval stage (Adán C, et al. 2008).

Similar results were obtained in *Tfb1m* knock-out mice, which resulted in embryo lethality. Heart specific *Tfb1m*$^{-/-}$ mice resulted in loss of mitochondrial protein translation, impairment on the mitochondrial ribosomal assembly and lack of adenine dimethylation of the rRNA of the small mitochondrial ribosomal subunit (Metodiev MD, et al. 2009).

2.4.3 Mitochondrial transcription

Mitochondrial DNA transcription initiation starts at the promoters located in each strand, the light strand promoter (LSP) and the heavy stand promoter (HSP). The majority of the genes are transcribed from the heavy strand and, in general, as polycistronic precursors (Montoya J, et al.1983). H-strand transcription gives rise to 10 mRNAs encoding 12 polypeptides, 2 rRNAs and 14 tRNAs. The mRNA presents two overlapping reading frames ND4L-ND4 and A8-A6. The L-strand encodes the remaining mRNA, (ND6), and 8 tRNAs. A second promoter, involved in the high rate synthesis of rRNA, is proposed to be located in the heavy strand (Montoya J, et al. 1983). Some of the known enzymatic proteins responsible for mitochondrial transcription are: a mitochondrial RNA polymerase (POLRMT) and TFAM (mitochondrial transcription factor A) (reviewed in Gaspari M, et al. 2004).

Mitochondrial transcription termination is proposed to be dependent on the mitochondrial transcription termination factor (mTERF) which possesses many binding sequences within the mitochondrial genome, notably at the 16S rRNA tRNA$^{Leu(UUR)}$ gene boundary, or just upstream of the $tRNA^{Phe}$ coding gene. mTERF is proposed to mediate transcription termination only at the L-strand. Recently, three novel mTERF related genes have been identified in vertebrates and they code for proteins homologous to mTERF. The new proteins have been called mTERF 2, 3 and 4. However the exact roles in mitochondrial transcription of the mTERF homologous proteins are not clear (Hyvärinen AK, et al. 2007; reviewed in Falkenberg M, et al. 2007).

2.4.4 mtDNA Replication

Mitochondrial DNA is proposed to replicate via two models. A first replication model, mainly based on results from the electron microscopy and 5' end mapping (reviewed in Clayton DA, 2003) proposed that the two mtDNA strands replicate asymmetrically using an asynchronous mechanism. Each strand contains one replication origin. MtDNA replication starts from the leading (heavy) strand, at the H-strand replication origin (O_H), which is also proposed to form the the D-loop, and it continues via strand displacement unidirectionally until two-thirds of the circular mtDNA is copied. Only once the replication fork passes the origin of replication of the light strand (O_L) is lagging-strand synthesis initiated. Following the synthesis, the two new strands are ligated to form the closed double stranded mtDNA.

The second proposed replication model is mainly based on 2D-gel electrophoretic analysis, which utilizes isolated mtDNA digested with appropriate restriction enzymes. The interpretation of the migration of mtDNA fragments in the 2 dimensional electrophoresis suggests how mtDNA replication occurs (Holt IJ, et al. 2000). According to this model, proposed by Holt and Jacobs, replication starts from a single origin region, close to O_H and proceeds unidirectionally or bidirectionally with or without RNA incorporation. The leading and lagging strands are synthesized simultaneously. Both mtDNA replication models can be found. However, under certain conditions, one mode might be more predominant than the other.

2.5 Nucleus-mitochondria signalling

Mitochondria cover many important roles within a cell: energy production, anabolic metabolism or apoptosis to control and activate programmed cell death. Mitochondria also mediate many cellular signaling pathways e.g. through calcium homeostasis regulation (reviewed in Ryan M, 2007).

Mitochondrial dysfunction is associated with a broad range of inherited and acquired human diseases including cancer and ageing-related processes, characterized by extensive gene expression changes. Mitochondrial associated diseases manifest generally in a tissue specific manner, being more severe in cells with high metabolic activities, such as neurons and muscle cells (reviewed in Wallace DC, 2005). Although mtDNA can code for few proteins, around one thousand proteins are estimated to be in a mitochondrion (Smith AC & Robinson AJ, 2009). Thus the nuclear genome encodes most of the mitochondrial proteins, as previously seen. However, many of these proteins still remain unknown. The biogenesis and function of mitochondria depend on the coordinated expression of the nuclear and mitochondrial genomes. The signaling network between the two genomes has to be also “flexible” to ensure a correct mitochondrial functionality according to the cell’s needs, i.e. the ability to respond to external stimuli such as changes of the environmental temperature (Puigserver P, et al. 1998), diet changes, caloric intake modifications (Yoon JC, et al. 2001) and exercise, probably as an adaptation to better use the available oxygen (Norrbom J. et al. 2004).

The communication network coordinating the activities of the two genomes can be distinguished in anterograde (nucleus to mitochondria) and retrograde (mitochondria to nucleus) signals. The anterograde mechanism consists of the modulation of gene expression in mitochondria in response to endogenous or external stimuli perceived by the nucleus, thus the signal goes from nucleus to mitochondria. The retrograde signals originate within mitochondria and they modulate the nuclear gene expressions which then in turn influences the anterograde pathway (reviewed in Poyton RO & McEwen JE, 1996). The pathways involved in retrograde signaling are important mediators that can modulate nuclear gene expression in order to compensate mitochondrial dysfunction which might arise, for example, during ageing (reviewed in Jazwinski SM, 2005).

Although the general process of mitochondrial signaling is conserved among eukaryotes such as in yeast and in humans, the exact molecular mechanisms by which signaling and signal transduction occur do not seem to be conserved (reviewed in Liu Z & Butow RA, 2006). Mitochondrial signaling is so far well studied and characterized in yeast, but still poorly understood in humans (reviewed in Liu Z & Butow RA, 2006). Yeast represents a useful model to study the consequences of mitochondrial dysfunction since it can grow well even without mtDNA, thus without respiratory-competent mitochondria, using glucose as a carbon source via glycolysis (Sekito T, et al. 2000). Through the comparative analysis of transcriptional profiles of respiratory-competent versus respiratory-deficient yeast cells, it has been possible to identify many components involved in mitochondrial signaling. Mitochondrial dysfunction can also be induced by treating yeasts with toxins, such as inhibitors of the mitochondrial electron transport chain (antymycin, rotenone, oligomycin) or the uncoupler of the proton gradient CCCP (reviewed in Butow RA & Avadhani NG, 2004). Nuclear-encoded transcripts responding to mtDNA defects can be sub-classified into two classes. The first class includes transcripts that respond to general mitochondrial respiratory capacity, while the second class includes transcripts that respond to mtDNA mutations (reviewed in Liu Z & Butow RA, 2006). Among the factors identified in mitochondrial signaling, the *CIT2* gene product appears to be a key element in retrograde signaling, and its expression can be increased up to 30 fold during mitochondrial dysfunction (Liao XS, et al. 1991). The identification of *trans*-acting elements regulating *CIT2* expression has identified other components of signaling that can act as positive or negative regulators in the retrograde pathways. Some positive acting factors are Rtg1p, Rtg2p and Rtg3p. Some negative acting factors are Mks1p, Lst8p, and a subset of 14-3-3 proteins (reviewed in Liu Z & Butow RA, 2006). Lst8p identification in retrograde regulation implies also the involvement of TOR in mitochondrial signaling (see below).

The expression of most eukaryotic genes is controlled at the level of transcription through *trans*-acting regulatory proteins (transcription factors). Each transcription factor can recognize a specific nucleotide sequence, a *cis*-acting sequence, within the promoter region or several thousands of nucleotides far from it, and the protein factor-DNA interaction modulates the expression of a gene (reviewed in Green MR, 2005). The different transcription factors regulate sets of genes whose expression can be stimulated or suppressed depending on the cell cycle state and even in a tissue-specific manner. Despite recent extensive investigations, there is no evidence of a single common regulatory element capable of coordinating the expression of the majority of the genes encoding for components of the respiratory chain and other mitochondrial proteins. However some elements are recurrently found involved in the gene expression regulation of such genes, like NRF-1, NRF-2, PPARα , ERRA (estrogen-related receptor) binding factors or PGC-1 co-activators in mammals (Van Waveren C & Moraes CT, 2008). In yeast, the CCAAT box-binding transcription factor Hap2/3/5, the homologue of mammalian NF-Y, appears to be the main activator of transcription of nuclear genes involved in mitochondrial biogenesis and OXPHOS (Buschlen S, et al. 2003; Lascaris R, et al. 2003).

2.5.1 Nuclear Respiratory Factors: NRF-1 and 2

So far, only a few *cis*-acting elements, transcription factors and coactivators, have been shown to have a regulatory role in the expression of a subset of nuclear genes involved in mitochondrial functions. NRF-1 (nuclear respiratory factor 1) was the first transcription factor to be identified as a functional regulator of the promoter activity of many nuclear genes governing mitochondrial maintenance and respiratory function. The expression of such genes can be directly or indirectly regulated by NRF-1 (reviewed in Scarpulla RC, 2008). NRF-1 recognizes and activates promoters of genes encoding key components of the mtDNA transcription machinery such as TFAM, both TFBm isoform genes and POLRMT (Cam H, et al. 2004). NRF-1 binds as a homodimer to its recognition site, which is present in the promoter of many OXPHOS encoding genes and which is proposed to be YGCGCAYGCGCR where Y and R represent respectively pyrimidine nucleotide and purine nucleotide (reviewed in Scarpulla RC. 2008). The DNA-binding and *trans*-activation functions of NRF-1 are enhanced by the phosphorylation at its multiple serine residues (Gugneja S & Scarpulla RC, 1997; Herzig RP, et al. 2000). PGC-1α coactivator, as well as steroid hormones, act as *trans* activators of NRF-1 target genes (reviewed in Scarpulla RC, 2008; Scheller K & Sekeris CE, 2003). NRF-1 also regulates the expression of key enzymes of the heme biosynthetic pathway, notably 5-aminolevulinate synthase (Braidotti G & Borthwick IA, 1993) and uroporphyrinogen III synthase (Aizencang GI, et al. 2000). It also controls the import of nuclear encoded proteins by activating the expression of TOMM20 and TOMM34, key subunits of the outer membrane multisubunit receptor complex, TOM, which translocates proteins into the intermembrane space (Blesa JR, et al. 2007; Blesa JR, et al. 2008). In mouse, NRF-1 regulates also the expression of Cox17, a protein involved in the assembly of cytochrome oxidase (Takahashi Y, et al. 2002). Due to its role in regulating the expression of components of the protein import and assembly machinery, NRF-1 provides a strategic control over mitochondrial biogenesis and respiratory function (reviewed in Scarpulla RC, 2008). Further studies, based on induced changes in basal metabolism, such as exercise training in rats, confirmed the role of NRF-1 in nucleus-mitochondria coordination according to cell needs. Exercise training resulted in the up-regulation of NRF-1 and PGC-1α in rat muscle, as an adaptive response to increased energy demand (Baar K, et al. 2002). mtDNA depletion in cells caused a parallel increase in NRF-1 and TFAM mRNAs in response to oxidative stress (Miranda S, et al.1999). TFAM and NRF-1 proteins and mRNAs are increased in skeletal muscle of aged subjects probably as a compensatory response to age-related reductions in energy metabolism (reviewed in Scarpulla RC, 2008). Further results confirm the critical role of NRF-1 in cellular processes, based upon the finding of the early embryonic lethality of mouse NRF-1 knockout embryos (Huo L & Scarpulla RC, 2001). NRF-1 binding factor is found also in organisms other than humans and mice, including sea urchins, *Drosophila*, chickens and zebrafish, but apparently not in yeast (reviewed in Scarpulla RC, 2002).

NRF-2 (nuclear respiratory factor-2) is a nuclear DNA-binding factor linked to mitochondrial function (reviewed in Scarpulla RC, 1997) involved in the activation of the expression of the 10 nuclear encoded cytochrome oxidase subunits and other respiratory genes (Virbasius JV, et al. 1993; reviewed in Scarpulla RC, 2002). NRF-2 comprises five subunits, a subunit α which binds directly to the DNA, and four other subunits β_1, β_2, γ_1 and γ_2. The NRF-2 binding site presents a GGAA core motif (reviewed in Scarpulla RC, 2008). NRF-2 activity is regulated similarly as seen for NRF-1, with PGC-1α acting as a co-activator (Hsieh YC, et al. 2005).

The essential role of NRF-2 was shown by a mouse knock-out model of GABPα, the mouse homolog of human NRF-2α. GABPα knock out mice presented a peri-implantation lethal phenotype (Ristevski S, et al. 2004). Other genes related to the respiratory chain machinery are regulated by NRF-2, among them TFAM and both TFB isoforms (Rantanen A, et al. 2001; McCulloch V, et al. 2002). The NRF-2 binding site can be present in promoters that also contain a NRF-1 site. Some promoters can clearly be regulated by both NRF-1 and NRF-2, as supported *in vivo* for human TFAM and TFB (-1M and -2M) by chromatin immunoprecipitation. Such double promoter activity regulation control by NRFs might be required to ensure correct coordination of the expression of respiratory subunits (Gleyzer N, et al. 2005; Virbasius JV & Scarpulla RC, 1994).

2.5.2 Retrograde calcium signaling

In mammals, mitochondria have a critical role in calcium storage and homeostasis. They do this by sequestering ions released from intracellular stores or following increased calcium uptake across the plasma membrane. Mitochondria can release calcium into the cytosol or into specific subcellular regions as part of the general process of intracellular traffic and signaling (reviewed in Smaili SS, et al. 2000; Butow RA & Avadhani NG, 2004).

Genetic alterations, mutations or mtDNA depletion and metabolic stresses, can cause the disruption of the mitochondrial $\Delta\psi$ (membrane potential) resulting in an abnormal increase in calcium levels in the cytosol. Metabolic stress can be induced by many factors, such as ionophores, ROS increase, drugs, and hypoxia. A non-physiological increase of calcium in the cytosol activates the calcineurin pathway and various calcium dependent kinases, resulting in dramatic changes in nuclear gene expression by altering cellular physiological functions. This may result in cell death and/or trigger pathologies such as cardiac arrhythmias and heart failure (Zayzafoon M, et al. 2006; reviewed in West DJ & Williams AJ, 2007).

Calcium entry into mitochondria is physiologically regulated by voltage-dependent channels located in the outer and inner membranes (Gincel D, 2001). The energy required for calcium ion entry into mitochondria is supplied by the membrane potential which is maintained by the proton gradient created by the electron transport chain. Appropriate calcium levels in mitochondria and cytosol are critical for cell survival and programmed cell death, since correct concentrations of the ions act as positive activators for OXPHOS, stimulating ATP production. However elevated concentrations activate uncontrolled pathways which lead to pathological processes resulting, as a last step, in cell death (reviewed in Brookes PS, et al. 2004). Therefore, at appropriate concentrations, calcium stimulates the OXPHOS machinery, activating enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase and ATP synthase, and thus increasing the ATP production rate in beating rat heart (Das AM & Harris DA, 1990). A pathological calcium level alteration leads to a cascade of pathological events such as ROS generation, cytochrome *c* release, and apoptosis (reviewed in Brookes PS, et al. 2004).

2.5.3 Reactive Oxygen Species: ROS

The term ROS (reactive oxygen species) describes reactive oxygen species derived from an incomplete reduction of oxygen. It includes the superoxide radical ($\mathbf{O_2^{\cdot -}}$), hydrogen peroxide ($\mathbf{H_2O_2}$), and the hydroxyl radical ($\mathbf{HO^{\cdot}}$). The only very reactive species among them is the hydroxyl radical, which has an indiscriminate reactivity and is considered to be responsible for most ROS-associated damage to biological molecules, including DNA (reviewed in Scheffler IE 1999). The hydroxyl radical, being uncharged, can pass through membranes where it can induce lipid peroxidation, thus damaging cell's compartmentations. The superoxide anion and hydrogen peroxide have a limited reactivity and, as shown in *Escherichia coli*, they exhibit preferential targeting; the superoxide anion has a strong reactivity with iron-sulfur clusters while hydrogen peroxide reacts with some Cysteine residues, thus resulting in thiol oxidation (reviewed in D'Autréaux B & Toledano MB, 2007).

The major risks from the later two species are represented by the production of hydroxyl radical in the following reactions.

$2\ O_2^{\cdot-} + 2H^+ = O_2 + H_2O_2$ reaction 1
$O_2^{\cdot-} + Fe(III) = O_2 + Fe(II)$ reaction 2
$Fe(II) + H_2O_2 = Fe(III) + OH^- + OH^\cdot$ reaction 3
Summing reactions 2 and 3 yields:
$O_2^{\cdot-} + H_2O_2 + H^+ = O_2 + H_2O + OH^\cdot$ reaction 4

Reactions 2 and 3 need the presence of redox metals. Reaction 3 is the Fenton reaction. The relatively low reactivity of hydrogen peroxide by itself can be enhanced by the presence of redox metals which are ubiquitously distributed within the cell (reviewed in Scheffler IE. 1999). Due to the danger of the above reactions, triggered by Fe(III) and Cu(I), which produces hydroxyl radical, the cell has developed a mechanism to reduce such potential risk by sequestering and localizing the "free" metal ions, so that in the worse case of reaction with superoxide radical the damage will be limited (reviewed in Chiancone E, et al. 2004). In normal metabolic processes, in cells, most of the ROS, in the form of the superoxide radical, is produced by the mitochondrial electron transport chain, which leaks a few electron directly to O_2, thus reducing it only in part (reviewed in Chan PH, 2001). ROS production from the electron transport chain occurs mainly in complexes III and I (reviewed in Jezek P & Hlavatá L, 2005). Since ROS is continuously produced during normal metabolism, aerobic organisms have developed potent protective mechanisms to keep ROS production under control. In mammals, one of the most important antioxidant enzymes is the manganese superoxide dismutase (MnSOD), which catalyzes the reaction of two superoxide anions with two protons to produce H_2O_2, accelerating the dismutase reaction (McCord JM & Fridovich I, 1969).

$2O_2^{\cdot-} + 2H^+ = H_2O_2 + O_2$

Superoxide radicals can be produced, as well, by other enzymes. Xanthine oxidase, present in the cytosol and in circulating blood, transfers electron to NAD+. However a pathological situation, such as during ischemia and reperfusion, the active site can be oxidized and consequently produces the superoxide radical (White CR, 1996; reviewed in Ullrich V & Bachschmid M, 2000). ROS species, in the form of the superoxide radical, are also produced in animals and plants by activated phagocytic white cells (neutrophils, macrophages, monocytes) during host defense, by a NADPH oxidase. The superoxide radical is then converted to hydrogen peroxide and hydroxyl radicals which kill the microbes prior to phagocytosis (reviewed in Iriti M & Faoro F, 2007).

Some metabolic enzymes also produce ROS during their normal function or when there is not enough substrate, among them: cytochrome P450s, Nitric oxide synthases, and cyclooxygenase (Liu L, et al. 2001; Xia Y, et al. 1996; Pomposiello S, et al. 1999). There are three isoforms of the metalloenzyme superoxide dismutase (SOD), all three of them nuclear-encoded. Although they catalyze the same reaction, they have different primary structures and three different cellular localizations, which are used to name the three SOD genes in higher eukaryotes (reviewed in Johnson F & Giulivi C, 2005). One isoform, the manganese-dependent SOD2, is localized within the mitochondrial matrix. The two other isoforms Cu/Zn SOD1 and Cu/Zn SOD3 are found respectively in the cytosol and extracellular space (reviewed in Culotta VC, et al. 2006). Knock out *Sod2*$^{-/-}$ mice die within a week, whereas knockout *Sod*1$^{-/-}$ or *Sod*3$^{-/-}$ mice are vital, but more sensitive to neuronal injury or hyperoxia (reviewed in Li Y, et al. 1995; Reaume AG, et al. 1996; Carlsson LM, et al. 1995).

Overexpressing the human SOD2 in mice resulted in neuromuscular abnormalities, similar to that seen in Down syndrome patients, whose tissues present an increase in Cu/Zn SOD levels. These data suggest that ROS levels have to remain at a certain threshold within cells. High amounts of ROS have deleterious consequences. However, levels too low are not healthy either (reviewed in Scheffler IE, 1999). Clinical trials have attempted to reduce ROS levels in trauma patients presenting severe head injury, at least taking as a baseline reference for oxidative stress the extent of plasma lipoperoxidation.

Enteral feeding with antioxidant vitamin C and E had only a small beneficial outcome (Crimi E, et al. 2004). ROS seems to be involved in many redox signaling processes such as cell cycle control, cell proliferation, apoptosis, metalloproteinase function, oxygen sensing and transcription factor activation (reviewed in Covarrubias L, et al. 2008).

2.5.3.1 ROS and signaling

Although ROS has always been considered highly toxic, there is now evidence of a ROS signaling influence in fundamental cellular redox reactions and other processes, including the action of growth factors. Angiotensin stimulates vascular smooth muscle growth upon increase of the superoxide radical, which then dismutates to hydrogen peroxide which may also act directly to promote cell proliferation. Through the regulation of the transcription factor NFkB, the superoxide anion seems to regulate the intracellular signaling of tumor necrosis factor α. Some of the signaling reactions mediated by ROS involve the oxidation of cysteine residues and formation of -S-S- bonds, thus conferring changes in protein conformation, which can then activate or inhibit certain pathways (reviewed in Magder S, 2006).

2.5.4 PPARgamma coactivator-1: PGC-1

The transcription factor PGC-1 is considered a master key regulatory element for mitochondrial biogenesis, acting as a coactivator of many other transcription factors which in turn regulate the expression of genes involved in mitochondrial function (reviewed in Lin J, et al. 2005). Two isoforms of PGC-1 are found: α and β. PGC-1 interacts with other proteins, such as CREB-binding protein, p300 and histone acetyl transferase, which activate dynamic chromatin remodeling processes, thus modulating transcription. PGC-1 is also involved in the corregulation of mRNA splicing and it activates the steroid hormone receptor associated protein TRAP/DRIP (reviewed in Scarpulla RC, 2006). PGC-1 is thus involved in the coordinate regulation of transcription and splicing in response to metabolic stimuli (reviewed in Knutti D & Kralli A, 2001).

The transcriptional activation of PGC-1 is highly modulated by external stimuli in a tissue specific manner. Cold exposure stimulates PGC-1 induction in brown adipose tissue. PGC-1 is induced in skeletal muscle following exercise. In fasted liver, PGC-1 is induced and it activates gluconeogenic and fatty acid oxidation genes (reviewed in Rodgers JT, et al. 2008). The PGC-1 level seems to be directly correlated with the number of mitochondria within a cell. Overexpressing PGC-1 in cell culture resulted in an activation of many genes involved in oxidative metabolism (Pirinen E, et al. 2007). In respiration, it increased proteins import into the mitochondria, and it boosted energy substrate utilization. Through the regulation of NRF-1 and NRF-2, PGC-1 can corregulate a number of genes important for mitochondrial functions. The PGC-1 level is also correlated with changes in intracellular calcium level via the calcium/calmodulin dependent protein kinase, which in turn is also involved in ROS signaling (reviewed in Ryan MT & Hoogenraad NJ, 2007).

2.5.5 CREB (cAMP response element-binding protein)

cAMP (cyclic adenosine 3′,5′-monophosphate) is a second messenger involved in many complex processes related to energy metabolism, cell proliferation, differentiation, adaptation, and survival through the modulation of transcription of target genes (reviewed in Szaszák M, et al. 2008). The induction of gene expression by cAMP is believed to be mediated by CREB, which binds to the specific sequence 5′-TGACGTCA-3´ present in the promoter of many cAMP-responsive genes (West AE, et al. 2001). CREB is a ubiquitously expressed nuclear transcription factor that is activated by several extracellular stimuli (reviewed in West AE, et al. 2001). Bioinformatic studies have estimated that half of the promoters containing a CREB binding site also contain TATA boxes, which are necessary for an optimal cAMP response (Zhang X, et al. 2005). The hydrolysis of cAMP to 5´AMP represents the signal termination and is catalyzed by cyclic nucleotide phosphodiesterases (PDEs). CREB transcriptional activity is normally enhanced by its phosphorylation at serine-133 mediated, by cAMP-dependent protein kinase (PKA) in response to certain stimuli, although not all the stimuli that induce the phosphorylation of serine-133 also activate CREB. However, CREB activity can be activated also by PKA-independent mechanisms. Mitogen/stess-activated kinase *Msk1* is proposed to be the major cAMP-activated serine-133 kinase in the brain during development. Also an increase in intracellular calcium level results in CREB phosphorylation at serine-133 by calmodulin-dependent protein kinase I, II, and IV (reviewed in Sands WA & Palmer TM, 2008). Some of the stimuli inducing CREB phosphorylation and activation are: growth factor signalling, steroid hormon signalling, neurosignalling, cytokines, NO and oxidative stress, immune cell signalling, enviromental cell signalling, and intracellular calcium signalling (reviewed in Johannessen M, et al. 2004).

Mutation of serine-133 to alanine, which is not phosphorylated, abolishes the increase in CREB transcriptional activity. This dominant-negative mutant form of CREB can still bind to the recognition site and thus prevents the binding of wild-type CREB (Gonzalez GA & Montminy MR, 1989). CREB is dephosphorylated by several phosphatases, such as the serine-threonine protein phosphatase 2A (PP2A) in hepatocyties. PP1 is the major CREB phosphatase in NIH3T3 cells (reviewed in Sands WA & Palmer TM, 2008). The *in vivo* functional role of CREB was studied in transgenic mice. Overexpressing the dominant negative CREB in pituitary gland induced atrophy of the gland, causing growth retardation in mice. Mice with targeted deletion of the CREB gene exhibit long term memory alteration (reviewed in Ichiki T, 2006). Based on recent data, CREB is proposed to have an important function in antidepressant treatments, which upregulate and activate CREB particularly in the hippocampus (reviewed in Gass P & Riva MA, 2007). However, CREB knock-out mice have almost normal phenotype, probably due to other compensatory mechanisms, such as the increase of expression of the cAMP response element modulator CREM, which is a gene with a similar activity to CREB, but proposed to have more tissue- specific functions (reviewed in Ichiki T, 2006). CREB deficient mice, created by gene deletion or by overexpression of a dominant-negative form of CREB, suffers fasting hyperglycemia and have reduced expression of gluconeogenic genes, which are normally activated by CREB through the nuclear receptor coactivator PGC-1. This suggests that CREB and PGC-1 might have a role in the pathogenesis of type II diabetes (reviewed in Ichiki T, 2006).

2.5.6 mTOR

Rapamycin, an FDA approved drug, was initially used as an anti-fungal drug and later as an immunosuppressant for organ transplantation and in chemotherapy. It is a potent anti-proliferative agent. The target genes of rapamycin were first identified in yeast and named TOR1 and TOR2. They allowed cells mutant for the two genes to continue growing under rapamycin treatment (reviewed in Yang Q & Guan KL, 2007). mTOR (mammalian target of rapamycin), the mammalian homolog of yeast TOR, is an evolutionarily conserved serine-threonine protein kinase. mTOR signaling is activated by hormones, growth factors, amino acids and cellular energy status (reviewed in Wang X & Proud CG, 2006). mTOR is part of two distinct multi-protein complexes, mTORC1 and mTORC2. mTORC1 is a heterotrimeric protein kinase which includes the mTOR catalytic subunit, as well as Raptor (regulatory associated protein of mTOR) and mLT8. Raptor is proposed to have a role in the assembly of mTOR, being involved in sensing and activating mTOR in response to nutrients and other stimuli (reviewed in Yang Q & Guan KL, 2007).

mTORC2 components are mTOR, mLST8, Rictor (rapamycin-independent companion of mTOR) and mSin1 (mitogen-activated protein-kinase-associated protein1). Both Rictor and mSin1 are necessary for the phosphorylation of Akt protein kinase B mediated by mTORC2. While still poorly understood, Rictor and mSin1 are proposed to be involved in actin organization and to mediate phosphorylation and activation of Akt, which is thus activated in response to growth factor signaling (reviewed in Lian J, et al. 2008; Rosner M et al. 2008). mTOR activity seems to be linked also to mitochondrial function, since mitochondrial dysfunction or oxidative stress inhibits mTOR activity. However, the pathway linking mitochondria to mTOR is still unknown (reviewed in Butow RA & Avadhani NG, 2004).

mTOR functions as an important control step in cell growth and proliferation, by regulating the mRNA translational machinery. mTOR links together protein synthesis regulation, signals from nutrients, growth factors and cellular energy metabolism. mRNA translation represents thus an important control step, since it consumes amino acids (reviewed in Kimball SR & Jefferson LS, 2006). In multicellular organisms, cell growth control determines also the overall organ and body size (reviewed in Schmelzle T & Hall MN, 2000).

The mTOR-based response to nutrient and cellular energetic status, seems to be mostly influenced by cellular amino acid level, especially by leucine, which is mostly taken in with food. Leucine, in particular, can stimulate translation initiation. Integrating signals from nutrient and growth factors mTOR can stimulate cell growth. mTOR functions also in stimulating mRNA translation (reviewed in Kimball SR & Jefferson LS, 2006).

A low energetic state corresponds to a low mTOR activity. mTOR signaling plays an important role in the brain, where it can influence food intake, which can be inhibited by elevated protein concentration in the brain (Cota D, et al. 2006). The essential role of the mTOR gene was shown by mTOR knockout mice which die *in utero* shortly after being implanted (Gangloff YG, et al. 2004).

So far, a calorically restricted diet represents the only universal way to extend lifespan, as shown in different organisms including mammals. Deleting genes encoding components of the nutrient responsive TOR pathways, increases lifespan. Caloric restriction is proposed to increase lifespan at least partially by decreasing TOR activity (reviewed in Lian J, et al. 2008).

2.5.7 Signaling and disease

2.5.7.1 Cancer

The possible link between mitochondria and cancer was first hypothesized by Warburg, who proposed that proliferating cancer cells use glycolysis more often than respiration (reviewed in Warburg O, 1956; Warburg O, 1930). In cancer cells, mitochondrial mass is highly decreased, similarly to what found in an undifferentiated state such as in an embryonic cell. Despite the high efficiency of mitochondria in producing ATP, the glycolytic pathway ensures most of the energy demand to the highly proliferating cancer cells, thus bypassing the slow shuttling of NADH from the cytosol into mitochondria, which is the limiting step for mitochondrial energy production (reviewed in Pedersen PL, 1978). Cancer cells appear like undifferentiated cells, and it has been observed that the more dramatic the loss of differentiation, the lower the mitochondrial capacity and the more aggressive the cancer. Mitochondrial functions are thus suppressed, and cannot control cell proliferation via apoptosis (reviewed in Halabe Bucay A, 2007). In many tumors, mtDNA presents mutations, which probably enhance tumorigenesis rather than initiate it. Direct evidence of the contributions of mutant mtDNA to cancer has been shown by the enhanced tumorigenesis of cybrid cells with mutant mtDNA compared to cybrid cells with wild-type mtDNA. When transplanted into nude mice, mutant mtDNA cybrids enhanced the tumor size (reviewed in Ohta S, 2006). mtDNA mutations affect mitochondrial functions and cause an abnormal ROS production increase that contributes to cancer progression (reviewed in Hüttemann M, et al. 2007).

An association between cancer and ROS production increase, due to mitochondrial dysfunction, was observed for mutations in nuclear genes encoding components of complex II, notably subunits B, C and D. Such mutations are considered to be the cause of paragangliomas, neoplasms that can be generally found in the head, neck, abdomen and thorax and are mainly benign tumors (reviewed in Favier J, et al. 2005). Functional abnormalities in complex II resulting in an increase ROS production might contribute also to Friedreich ataxia, a neurodegenerative disease caused by mutations in the nuclear frataxin gene. Frataxin may interfere with complex II assembly or structure, since it interacts directly with complex II (reviewed in Calabrese V, et al. 2005).

An increased ROS production from the electron transport chain is often found in neurodegenerative diseases such as Parkinsons, Alzheimers, Friedriech’s ataxia (FRDA) and multiple sclerosis (reviewed in Hüttemann M, et al. 2007). FRDA is caused by a deficiency in the frataxin protein (FXN) due to gene mutations or intronic hyperexpansion of a GAA triplet repeat. FXN is involved in intracellular iron metabolism such as biogenesis of heme group, iron homeostasis and iron chaperone activity (reviewed in Bencze KZ, 2006). FRDA is characterized by the degeneration of the

Purkinje neurons of the cerebellum. It results in an altered iron metabolism. Abnormal ion accumulation leads to a loss of iron-sulfur clusters, a suppressed oxidative phosphorylation and mitochondrial function in general, and increased oxidative stress (reviewed in Baron M, et al. 2007).

Parkinson's disease (PD) seems to be associated with a mitochondrial deficiency with an increase in oxidative stress affecting the neurons of the *substantia nigra* (reviewed in Forno LS, 1996). Many experiments, i.e. enzymatic measurements in cultured fibroblast and skeletal muscle cells from PD patients and cybrid cells, have shown a decrease in complex I activity. Complex I seems to be the main source of ROS. The PD degeneration is believed to be localized in dopaminergic neurons since cytosolic tyrosine hydroxylase and monoamine oxidase, while producing dopamine neurotransmitter, also seem to produce ROS (reviewed in Gandhi S & Wood NW, 2005). In PD, mtDNA presents rare mutations, but mtDNA deletions, which generate mitochondrial dysfuntion, are considered to be the main cause of the observed mitochondrial abnormalities (reviewed in Onyango IG, 2008).

2.5.7.2 Amyotrophic lateral sclerosis (ALS)

ALS is a neurodegenerative disease of the anterior horn cell of the spinal cord and cortical motor neurons. This progressive disease is characterized by a robust increase in ROS which produces damages in the neurons. The prime cause of the disease remains unknown. In patients with autosomal dominant ALS, the gene $Cu^{2}/Zn^{2+}SOD1$ (superoxide dismutase) presents mutations, however the enzymatic activity is retained. In ALS patients, mitochondria exhibit structural and functional abnormalities (reviewed in Pasinelli P & Brown RH. 2006). Bioenergetic deficiency, due to mtDNA deletions, is found in many neurodegenerative diseases, such as progressive external ophthalmoplegia (PEO) and Alzheimer's disease (AD) (reviewed in Chen X, et al. 2006). mtDNA deletions are consequences of mutations in nuclear genes encoding proteins involved in mtDNA maintenance; such as deoxyguanosine kinase, thymidine kinase, adenine nucleotide translocase, the mitochondrial helicase Twinkle and mitochondrial DNA polymerase γ (reviewed in Baron M. et al. 2007).

Mitochondrial dysfunction causes bioenergetic deficiency, resulting in a decreased production of ATP. However, ATP depletion alone doesn't seem the main cause of disease even in tissues with high energetic demands e.g. as proposed for Leber hereditary optic neuropathy (LHON). In LHON, complex I activity is slightly decreased and likely causes also small decrease in ATP production. Other inherited mutations strongly decreasing OXPHOS capacity can cause severe pathologies but not LHON. ROS increase, ATP depletion and modifications of nuclear and mitochondrial gene expression are proposed to be responsible for LHON pathogenesis (reviewed in Yen MY, et al. 2006). Mitochondrial functional abnormalities have often been shown to cause an increase in ROS production, which induces oxidative stress and produces deleterious consequences for the cell (reviewed in Brière JJ, et al. 2004).

2.5.7.3 Hypertension

Increased oxidative stress seems to correlate also with hypertension and congestive heart failure (reviewed in Puddu P, et al. 2008). Hypertension is a world-wide pathology, characterized by an increase of blood pressure, that in the long term can damage many organs and cause for example stroke and transient ischemic attack which can be fatal or permanently disable a person. The prime cause of hypertension pathogenesis is generally unknown. An increased oxidative stress status is found in patients with hypertension. The H_2O_2 level is highly increased in the plasma of hypertensive patients. Even normotensive subjects with a genetic risk of hypertension, based on family history, present a higher level of H_2O_2 than normotensive subjects without a family history of hypertension (reviewed in Puddu P, et al. 2008). This suggests a double correlation between oxidative stress-hypertension and a possible genetic predisposition. The ROS increase is probably a consequence of an upregulation and/or dysfunction in NADPH oxidase, which exhibited increased activity in failing human myocardium or in a guinea pig model of progressive pressure-overload cardiac hypertrophy (Lacy F, et al. 2000; Li JM, et al. 2002). NADPH oxidases are enzymes broadly present in many cell types and notably in neutrophils, where once activated they generate ROS for microbial killing prior to phagocytosis (reviewed in Seddon M, et al. 2007). Hypertensive patients present also a reduction in antioxidant defenses, which enhances the deleterious effects of ROS. Clinical trials based on antioxidant administration in hypertensive patients are inconclusive, since they could not always lower blood pressure. However a diet rich in fruits and vegetables could result in a decrease in blood pressure, considering the general beneficial effects of increased antioxidant levels in the body (reviewed in Paravicini TM & Touyz RM, 2008).

2.6 Mitochondria and Drugs

Due to the critical roles provided by mitochondria in cells, it is necessary also to consider how therapeutic drugs may affect mitochondrial functions. Many drugs and xenobiotics show as a side effect a mitochondrial toxicity, affecting various mitochondrial functions. However mitochondria could also represent a direct specific target for novel drugs aiming to compensate a mitochondrial dysfunction (reviewed in Scatena R, et al. 2007). Recently, “pro-apoptotic” anticancer drugs have been designed specifically to target mitochondria and reactivate mitochondrial functions, notably the apoptotic pathway, which is often suppressed in cancer, the therapeutic aim being to trigger apoptosis and necrosis (Ziedan NI, et al. 2008).

The chemical modifications carried out in the more alkaline mitochondrial matrix, can trap and accumulate xenobiotics and in the long term lead to mitochondrial dysfunction (reviewed in Wallace KB & Starkov AA, 2000). The nucleoside reverse transcriptase inhibitors (NRTIs) are lifelong drugs used in HIV therapy; however they cause a considerable mitochondrial toxicity. NRTIs are nucleoside analogs and thus can be used also by DNA polymerase γ, resulting in a premature chain termination during mtDNA replication. NRTIs seem to decrease the fidelity of DNA synthesis by DNA polymerase γ and to inhibit thymidine phosphorylation. The inhibition of the mtDNA replication machinery results in the depletion of mtDNA, disruption of the electron transport chain, ROS production increase and apoptosis (reviewed in Scruggs ER & Dirks Naylor AJ, 2008; Cherry CL, et al. 2005). In patients, NRTI drug treatment induces lipodystrophy, a disorder in fat metabolism characterized by obvious adipose tissue accumulation and metabolic abnormalities, such as insulin resistance, diabetes and hypertension (reviewed in Sattler FR, 2008). The cause of lipodystrophy is multifactorial and is proposed to originate from the side effects of antiretroviral medications, by genetic predisposition and also perhaps directly by acute HIV infection, as observed in some patients (reviewed in Sattler FR, 2008). Lipodystrophy may be the consequence of NRTI-induced mitochondrial dysfunction which impairs fatty acid oxidation and causes intracellular accumulation of triglycerides (reviewed in Esser S, et al. 2007). NRTIs inhibit also the activity of sterol-sensing transcription factors involved in fatty acid and glucose metabolism and necessary for adipocyte differentiation, such as SREBP (sterol response element binding protein) and PPARγ (peroxisome proliferator-activated receptor gamma; Lemoine M, et al. 2006). Some clinical manifestations of the mitochondrial toxicity of NRTIs are similar to inherited mitochondrial diseases (reviewed in Esser S et al. 2007).

Rather little is known about drugs interfering with the complexes of the electron transport chain. They can inhibit directly an enzyme, act as an electron acceptor or uncouple OXPHOS, resulting in mitochondrial deficiency and generally an increase of ROS.

Complex I: MPTP (1-methyl-4-phenyl-1-2-3-6-tetrahydropyridine) and its metabolite MPP+ inhibits complex I preferentially in dopaminergic neurons, in which MPP+ is taken up by the dopamine transporter. They induce the same clinical and neuropathological manifestations observed in idiopathic Parkinson’s disease i.e. loss of dopaminergic neurons at the *substantia nigra*, accumulation of neuromelanin in microglia and the extracellular matrix (reviewed in Beal MF, 2003; Javitch JA, et al. 1985).

Many other drugs are known to induce an inhibition of complex I resulting in a pathological status similar to inherited mitochondriopathies such as acute liver failure. Such drugs often present a similar backbone chemical structure. Among them we can find the so called fibrates (used to lower cholesterol) and thiazolidinediones (used in diabetes mellitus type2 treatment; Brunmair B, et al. 2004; Ghosh S, et al. 2007).

Complex II: Some of the drugs altering complex II function are the anthracyclines, which are used to treat a broad range of cancers (reviewed in Wallace KB & Starkov AA, 2000).

Chlorimipramine, a tricyclic antidepressant drug, is shown to inhibit complex III activity (Daley E, et al. 2005).

NO and ONOO- interact and inhibit complex IV and they affect many other mitochondrial functions (reviewed in Moncada S & Bolaños JP, 2006). The cellular level of NO is very delicate due to its broad effects. In physiological conditions it is required for important processes, such as host defense, cellular proliferation regulation, vasodilatation and neurotransmission, but in pathological conditions represented by an abnormal reduction or increased cellular level it has deleterious effects i.e. blockade of mitochondrial respiration, apoptosis, necrosis, oxidative stress and DNA damages (reviewed in Pannu R & Singh I, 2006).

NO is a gas endogenously produced in the cytosol and mitochondria by nitric oxide synthase enzymes (reviewed in Carreras MC & Poderoso JJ, 2007). Thus, pharmacological interventions aiming to modulate cellular NO level should be carefully dosed. NO modulator agents are used as therapeutic drugs to treat many diseases such as *angina pectoris*, coronary artery disease, hypertension, osteoporosis, gastrointestinal motility disorders, pregnancy-related disorders including premature delivery, pre-eclampsia, and erectile dysfunction in men (reviewed in Wimalawansa SJ, 2008). Therapeutic strategies include the use of NO-donors that directly or indirectly release NO and compounds that modulate the activity of nitric oxide enzymes (reviewed in Pannu R & Singh I, 2006). Some of the drugs that can enhance NO production are antihypertensive agents, in particular angiotensin-converting enzyme inhibitors, calcium channel blockers, some vasodilating beta-blockers and some statins. Some drugs which indirectly release NO are the organic nitrates nitroglycerin and nocoradil. Drugs directly releasing NO are diazeniumdiolates, sydnonimines, S-nitrosothiols and NO gas which is itself used in therapy (reviewed in Mason RP & Cockcroft JR, 2006). Some drugs such as cerivastatin, a member of the statins, used to lower cholesterol, can alter the interaction of cytochrome-c oxidase (complex IV) with the membrane or other components of the electron transport chain, inducing mitochondrial dysfunction (Nadanaciva S, et al. 2007). Menadione, vitamine K3, acts as an uncoupler by shunting electrons directly from the complex I to the complex IV giving rise to an increase in ROS level (Doughan AK & Dikalov SI, 2007). Doxorubicin or its metabolites can accept one electron from complex I and trigger a radical reaction cascade (reviewed in Wallace KB, 2003).

The widely used nonsteroidal anti-inflammatory drugs (NSAID) such as aspirin, diclofenac and nimesulide, can uncouple the oxidative phosphorilation by dissipating the proton gradient, resulting in ATP depletion and heat production in cultured hepatocytes, but at drug concentrations much higher than the therapeutic dosages. Although the incidence of NSAID hepatic incidents are very low (about 0.1 case per 100,000 treated patients) they cause severe liver pathology (reviewed in Boelsterli UA. 2002). Salicylic acid and its acetylated form, aspirin, can also inhibit α-ketoglutarate dehydrogenase in the tricaboxylic acid cycle (Nulton-Persson AC, et al. 2004). Propanolol, a local anesthetic, directly inhibits complex V (reviewed in Scatena R., et al. 2007). The mitochondrial mRNA translational apparatus can be impaired by bactericidal antibiotics such as chloramphenicol, aminoglycosides, tetracycline and oxazolidizones already at therapeutic doses due to the high structural similarities of the functionally critical regions of the mitochondrial and bacterial ribosomes, which are the target site for the antibiotics (reviewed in Tenson T & Mankin A, 2006). A prolonged protein synthesis inhibition results in mitochondriophaty with manifestations similar to those of inherited mitochondrial diseases, such as myelosuppression and hearing loss (reviewed in Wallace KB & Starkov AA, 2000; Fischel-Ghodsian N, et al. 2004). However, toxic effects of antibiotics are seen only in a limited number of patients, suggesting that the toxicity depends on the genetic variation between individuals. Some mitochondrial mutations appear to increase the similarity between mitochondrial and bacterial ribosomes and correlate with aminoglycoside induced hearing loss and potentially decreased protein synthesis (reviewed in Fischel-Ghodsian N, et al. 2004; Jacobs HT, 2003).

2.6.1 Mitochondria as therapeutic target

Since mitochondrial dysfunction is normally characterized by an increase in ROS production, a therapeutic intervention aiming to increase the antioxidant capacity in mitochondria could be beneficial.

Coenzyme Q10, a potent ROS scavenger targeting mitochondria, is used to treat inborn OXPHOS dysfunction. CoQ10 treatment results in an improvement in neurodegenerative disorders e.g. Alzheimers. Menadione, a precursor of vitamin K, is used to treat complex III dysfunction. There is extensive drug screening to find molecules that target mitochondria, which could be beneficial in the treatment of stroke or myocardial infarction. The increase of oxygen availability, following hypoxia, and reactivation of mitochondrial functions, is characterized by an overproduction of ROS (reviewed in Schwartz DR & Sack MN, 2008).

Although antioxidant therapy, notably with coenzyme Q, has shown some improvements in the treatment of pathologies characterized by an increase of mitochondrial oxidative stress, the major problem is that only a small proportion of the administrated agent is really entering the mitochondrial matrix (reviewed in Sheu SS, et al. 2006). Recently a compound called MitoQ has been synthesized, which can specifically accumulate several hundred-fold within the mitochondrial matrix compared to other antioxidants such as coenzymeQ. MitoQ accumulation in mitochondria is driven by the mitochondrial membrane potential due to its chemical physical properties which present a positive charge (reviewed in Cochemé HM, et al. 2007). MitoQ has antioxidant properties and is derived by the conjugation of the lipophilic triphenylphosphonium cation to a ubiquinone (reviewed in Cochemé HM, et al. 2007). Post-ischemic reperfusion, following a myocardial infarction, is characterized by a burst in mitochondrial ROS formation (reviewed in Di Lisa F, et al. 2007). In rat animal models of cardiac ischemia-reperfusion, MitoQ significantly decreased heart dysfunction (Adlam VJ, et al. 2005). A MitoQ Phase I clinical trial showed good pharmacokinetic behavior and tolerability and Phase II clinical trials against Parkinson’s disease will soon be completed. MitoQ trials against other pathologies, such as Friedreich’s ataxia are in process (reviewed in

Tauskela JS, 2007). Recently, mitochondria have been considered a possible target for anticancer drugs. The idea is to create drugs that could reactivate the mitochondrial apoptotic pathway, which is suppressed in highly proliferating cancer cells. Drugs specifically targeting mitochondria in cancer could be developed, exploiting the negative membrane potential which is more elevated in undifferentiated cancer cells (reviewed in Scatena R. et al. 2007).

2.7 Bidirectional promoters

In the mammalian genome, 11% of the genes are estimated to be arranged head to head with their transcription start site (TSS) separated by less than 1 kb by an intergenic region, and thus designated as bidirectional promoters. The two genes in such pairs are divergently transcribed. The TSS of one gene is on the “plus” strand while the TSS of the other gene is on the “minus” strand. Bidirectional promoters are believed to co-regulate the expression of both genes through shared regulatory elements. However, the function of the two encoded proteins is not always clearly correlated. Gene expression can be regulated also by a canonical or unidirectional promoter which controls the expression of only one gene (Adachi N & Lieber MR, 2002; Lin JM, et al. 2007).

Gene transcription occurs through RNA polymerase II, whose access to the promoter site is regulated by many other mediating factors. Some factors among them, normally multiprotein complexes, called transcription factors, regulate the expression level of a gene, recognizing and binding specific short sequences within the promoter. Following the interaction with its binding site, a transcription factor can activate or repress the expression of a gene (reviewed in Boeger H, et al. 2005). Some transcription factors can recognize a common core sequence, such as for the 5 nt of the CCAAT box, but the flanking regions can shift the affinity in favour of one binding factor over others (reviewed in Ramji DP & Foka P, 2002).

In general the activity of any given promoter is regulated by several transcription factors. This allows a tight control over gene expression depending on the demands placed upon the cell, e.g. as previously seen, the transcription factors NRF-1 and NRF-2 can modulate the expression of many genes, especially the ones encoding mitochondrial proteins, based on energetic demands (reviewed in Scarpulla RC, 2008). Often, a promoter, to be active, requires the presence of an enhancer. The enhancer, a specific sequence element generally located around several kb from the starting site of a gene, is recognized by an activator. Interaction with its binding site allows transcription initiation via chromatin remodeling (reviewed in Polikanov YS, et al. 2007).

The *cis*-acting binding sites regulating bidirectional or unidirectional promoters are essentially the same within a species. Some binding-factor proteins have very conserved amino acid sequence even among different species. The major binding sites found in eukaryote promoters are the TATA or CCAAT boxes; occasionally they are found together within the same promoter. In promoters containing a TATA box, the CCAAT box is normally located -60 to 100 bp away from the transcription start site, while in TATA-less promoters, the CCAAT box is located at -40 to -80 bp from the transcription start site. Based on their ubiquitous distribution within genomes, the TATA box and CCAAT box are in general considered to be the main sites for regulating promoter activities. The TATA box is among the most widely found binding sites in eukaryotic promoters (reviewed in Juven-Gershon T, et al. 2008).

The TATA box is a conserved A-T- rich sequence in the DNA, normally located around 25 bp upstream from the start site of transcription. The TATA-binding protein (TBP) is a general transcription factor involved in initiation of transcription through RNA polymerase II. TBP interacts with TATA binding protein-associated factors (TAFs) to form the TFIID protein complex, which then facilitates the initiation of transcription (reviewed in Van Roon-Mom WM, et al. 2005). The CCAAT core binding site can be recognized by many multiprotein factors, especially NF-Y and the C/EBP transcription factor family (CCAAT/Enhancer Binding Protein), which includes C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ , C/EBPε and C/EBPζ (reviewed in Mantovani R,1998; Ramji DP & Foka P, 2002).

The C/EBP proposed ideal consensus site matches with 5´- RTTGCGYAAY-3´, where R is A or G, and Y is C or T. However it's not always the case, the binding site showed also substantial variations. C/EBPs present complex regulation patterns, tissues specificity and various roles during the different stages of tissue-organ development and differentiation. The cellular organization and function of C/EBPs appear complex, since the proteins can form heterodimers in all intrafamilial combinations and, with exception of C/EBPζ, interact with identical recognition sequence (reviewed in Ramji DP & Foka P, 2002).

The pentanucleotide CCAAT box is one of the most ubiquitous elements and it is found in around 30% of eukaryotic promoters. Within a promoter, more than one CCAAT box can be present, however the distance between CCAAT boxes is never less than 27 bp. The full binding site consists also of the flanking sequences of the CCAAT boxes which can shift the affinity in favour of one transcription factor over another. The NF-Y proposed ideal consensus site needs to be extended beyond the central pentanucleotide and it matches with 5´-C,G/A,G/A,C,C,A,A,T,C/G,A/G,C,A/C-3´ (reviewed in Mantovani R, 1999). NF-Y can interact with other functionally important elements, and thus initiate and/or modulate transcription. NF-Y has been shown to interact with the TFIID complex, and thus with the TATA-binding elements, and with the sterol regulatory elements (SREBPs), notably in promoters of genes involved in cholesterol metabolism (reviewed in Mantovani R, 1999; Frontini M, et al. 2001).

NF-Y consists of three subunits NF-YA, NF-YB and NF-YC all required for DNA interaction. However, only NF-YA is directly binding to the recognition site. NF-YA is the major subunit involved in regulating NF-Y function (Manni I, et al. 2008). Around 25% of the CCAAT boxes found in promoters are considered to be under the regulation of NF-Y, which is the main CCAAT box binding factor. NF-Y exhibits bifunctional activity, by enhancing or suppressing specific gene expression (Bernadt CT, et al. 2005; reviewed in Mantovani R, 1998). Canonical or bidirectional promoters can have multiple CCAAT boxes. In the *Mrps12/Sarsm* bidirectional promoter we identified an array of four CCAAT boxes involved in its regulation. Due to the unique ability of NF-Y to bind both CCAAT and ATTGG orientations on the coding strand, it can be considered as a good candidate transcription factor to govern bidirectional promoters (Figure 2.3: schematic representation of transcription initiation).

In the yeast *S. cerevisiae*, the CCAAT box-binding transcription factor Hap2/3/5, functional homologue of mammalian NF-Y, appears to be the main activator of transcription of nuclear genes involved in mitochondrial biogenesis and OXPHOS, such as the cytochromes, key components of mitochondrial translation apparatus and other genes which are activated by non-fermentable carbon sources (Buschlen S, et al. 2003; Pinkham JL & Guarente L, 1985; reviewed in Schüller HJ, 2003). In mammals, NF-Y regulates the expression of many genes related to aging and stress-related damage. Among them are the stress-response gene HSP70, histone H3, thymidine kinase, histone H2B.The overexpression of NF-YA, NF-YB and NF-YC subunits could rescue a pathological cytochrome c oxidase assembly mutant in the *SURF1* gene in human fibroblasts, thus demonstrating an important role of NF-Y in maintaining the OXPHOS system (Fontanesi F, et al. 2008). The mRNA and especially the protein level of NF-YA are decreased during aging. DNA damaging agents and growth stimulation increase the NF-YA protein level. NF-Y is involved in cellular oxidative stress conditions: it regulates catalase gene expression in both humans and mice (reviewed in Matuoka K & Chen KY, 2002; Luo D & Rando TA, 2003; Nenoi M., et al. 2001). Also C/EBP responds to cellular redox state, by controlling the expression of the *Cu/Zn SOD* gene in humans (Seo SJ, et al. 1996).

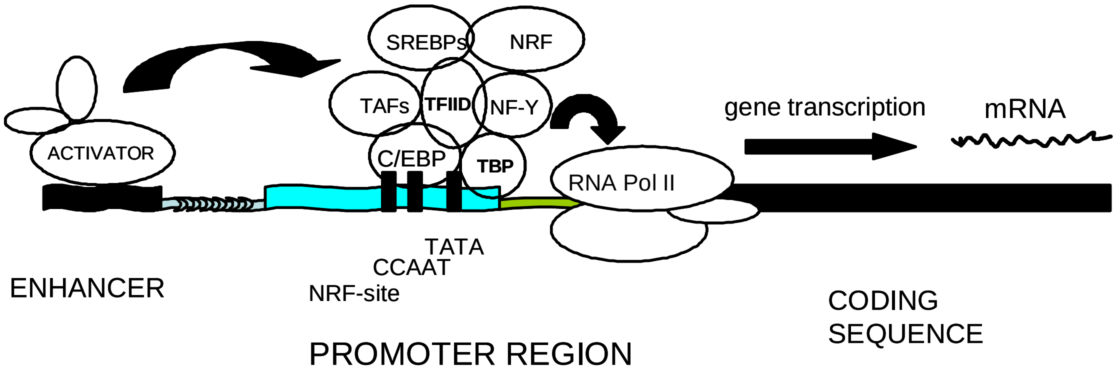


Figure 2.3. Schematic representation of transcription initiation: The activator interacts with its recognition site, enhancer, and thus permits the chromatin remodeling and relaxing. The activator-enhancer complex will then interact with multi-subunit complexes located on the promoter region. The multi-subunit complexes can then interact with RNA Polymerase II which initiates transcription by synthesizing mRNA. In the core promoter region several binding sites, such as TATA, CCAAT box and NRF-1 and 2 site, can interact with their specific protein-factors, TBP, NF-Y, C/EBP or NRF-1 and 2. The protein factor-DNA interaction activates RNA Pol II. Other protein-factors of the multi-subunit complex such as TFIID, TAFs or SREBPs can function on coordinating the multi-protein complex assembly or on modulating transcriptional level.

3. AIMS OF THE STUDY

Mrsp12 (mitoribosomal protein s12) and *Sarsm* (mitochondrial seryl-tRNA ligase) are nuclear genes coding for components of the mitochondrial translational machinery. Thus, the two genes appear to be involved in mitochondrial biogenesis and function. In human and mice, *Mrps12* and *Sarsm* genes are oppositely transcribed from a 250 bp intergenic region constituting a bidirectional promoter.

I addressed my studies to the following two issues:

Characterization of the function of the human and mouse *Mrps12/Sarsm* bidirectional promoter and identification of key regulatory elements governing its transcriptional activity.

Analyses of the response of *Mrps12/Sarsm* promoter to induced mitochondrial stress in different cultured cell-lines.

4. MATERIALS AND METHODS

4.1 Cells and cell culture

Mouse NIH 3T3 and C2C12 cells and human HEK293, U2OS and HeLa cells were cultured in DMEM (Cambrex) supplemented with 10% fetal bovine serum, containing 50 U/ml penicillin (Cambrex) and 50 μg/ml streptomycin (Cambrex). All cells were maintained at 37 °C in 5% CO_2, and passaged every 2–3 days as required.

4.2 RNA extraction

RNA was extracted from cells at 80% confluence using TRIzol reagent (Invitrogen) according to the manufacturer's recommended conditions, with resuspension in 30 μl nuclease-free water per 10 cm plate of cells. Treatment with 15 U RNase-free DNAse I (Amersham Biosciences) was carried out for 1 h at 37 °C in a final volume of 50 μl of manufacturer's recommended buffer. RNA was recovered by phenol–chloroform extraction; ethanol precipitation and finally resuspended in 30 μl nuclease-free water, and its purity and integrity were checked electrophoretically.

4.3 q-RT-PCR

First strand cDNA was synthesized from 5 μg cellular RNA using M-MuLV reverse Transcriptase (Fermentas) under manufacturer's recommended conditions, using 0.5 μg oligo(dT) primer. After treatment with boiled RNase (Roche) the cDNA was used in PCR in a 5-fold dilution series in the presence of SYBR Green (Qiagen) for fluorescent quantitation of the product, using the following gene-specific primers (all sequences shown as 5′–3′). For *Sarsm*, SARSM 8: CTTTCAGGGACCTTCCAGTCA and SARSM 4: CCGTCAAGATCTCCACCTGC; for *Hprt*, mHPRT_R1: CACAGGAC TA GAACACCTGC and mHPRT_F1: GTTGGATACAGGCCAGACTTTGT; for *Mrps12*, Mrps55: TTCCATGGCCACCCT GAACCAG and Mrps32: CAGCACTTGCGGTTGG CGGAG. Cycle parameters were: denaturation at 94 °C for 15 s (initial denaturation at 95 °C for 10 min), annealing for 10 s at 57 °C (for *Hprt*) or 55 °C for *Sarsm* and *Mrps12*, extension at 72 °C for 20 s, 55 cycles (Light cycler, Roche).

4.4 Reporter constructs

A dual luciferase reporter vector was constructed by recloning the entire coding sequence of firefly luciferase, including the SV-40 late poly(A) region, from the vector pGL3-basic (Promega) into the vector phRL-null (Promega), as an *NcoI–SalI* fragment. The resulting vector (pFRL) contains firefly and *Renilla* luciferase reporter genes oriented so that transcription is in opposite directions, with a unique *NcoI* site (CCATGG) between them, which provides the in-frame start codon in both directions. The *Mrps12-Sarsm* intergenic region was amplified with the following chimeric primers, using mouse or human genomic DNA clone as templates (*NcoI* restriction sites in bold, non-templated nucleotides underlined, all sequences 5′–3′),

Mrps12:<u>CATG**CCATGG**</u>CTCGCCGCCTGCAGCGTCCC,

Sarsm_F: <u>CATG**CCATGG**</u>CTTGGAGTGGAAACAAGAAGTCAC for mouse and RPMS12F_1: <u>CATG**CCATGG**</u>CCTGCCACCTGGGCCGTCCCTG. HsarsmF_1: <u>CATG**CCATGG**</u>CTTGGACCGGGAACAAGGCGG for the human. The PCR product was digested with *NcoI* and then cloned into *NcoI*-cut pFRL, generating clones in both orientations. To create a deletion series from the wild-type template, either of the same terminal primers was used together with the deletion primers indicated in Supplementary data of the published articles (I, II), with each product cloned into the *NcoI* site of pFRL using a similar strategy as for the wild-type promoters. To create point mutants of the dual reporter construct, a two-round mutagenic PCR strategy was employed, except for those cases where the mutations to be introduced were within 40 bp of the end of the intergenic region, and could thus be created in a single mutagenic step using modified terminal primers. In each case the final PCR product, following digestion with *NcoI*, was cloned into the *NcoI* site of pFRL. Every construct was verified by sequencing in both directions using the following primers: PhRL_R1: 5′-CCAGCACGTTCATTTGCTTGCAGC-3′ and PgL3_R1: 5′-CATCTTCCAGCGGATAGAATGG—3′

4.5 DNA transfections and reporter assays

Cells grown to 80% confluence were transfected in 6-well plates, using TransFectin Lipid Reagent (Bio-Rad) or FUGENE 6 (Roche, U2OS cells only), according to the manufacturer's instructions, with either 0.25 μg (HEK293 cells only) or 0.5 μg of DNA from each luciferase construct, plus the same amount of DNA of the β-galactosidase control vector. After 24 h of transfection Firefly and Renilla luciferase activities were assayed in cell lysates using the Dual Luciferase Reporter Assay System (Promega) and a BioORBIT 1254 luminova luminometer. Beta-galactosidase activity was determined in 50 μl aliquots of the same lysates by the addition of 50μl ONPG solution (2-nitrophenyl β-d-galactopyranoside) and 10 μl of 500 mM NaCl, 100 mM $MgCl_2$, 100 mM β-mercaptoethanol, with incubation at 37 °C for 1 h and measurement of A_{420}. Luciferase activities were normalized for transfection efficiency using β-galactosidase activity.

4.6 Drug treatments

FCCP (Sigma) dissolved in ethanol, antimycin (Sigma) plus rotenone (Sigma) dissolved in ethanol, thiamphenicol (Sigma) dissolved in DMSO or doxycycline (Sigma) dissolved in water, were added to cells at the concentrations indicated in the figures for 12 h prior to transfection, after which the medium was changed immediately before transfection. For prolonged exposure, the drugs were reintroduced to the culture medium 4 h after transfection. In trial experiments it was determined that the solvents used (ethanol and DMSO) had no effect on promoter activity. Thiamphenicol and doxycycline were always freshly prepared.

4.7 Mitochondrial ROS production

Mitochondrial ROS production was measured fluorimetrically, essentially as described by Mukhopadhyay M et al. (2007), using the mitochondrial superoxide-specific indicator MitoSOX™. Cells at 80% confluence were treated (or not) with mitochondrial inhibitors for 12 h, washed with fresh Opti-MEM medium (Invitrogen), then treated for 30 min at 37 °C with 1 ml of 5 μM MitoSOX™ Red (Invitrogen) in Opti-MEM medium. Cells were washed twice with PBS warmed to 37 °C, then resuspended in 600 μl PBS and analysed immediately by flow cytometry. MitoSOX was excited by laser at 488 nm, and the data were collected at 580 nm (FACSAria, BD Biosciences).

4.8 EMSA (Electrophoretic mobility shift assay)

Nuclear extracts were prepared from NIH 3T3, or HEK293 cells or mouse tissues. 1.5 g of freshly isolated tissue was homogenized with a Potter apparatus in 1ml buffer A (Buffer A: 10 mM Hepes pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5mM DTT, 100 ng/ml Aprotin; 5 ng/ml Leupeptin; 0.5 mM PMSF). The cell debris was removed by centrifugation and 500 μl of the supernatant was collected. $50x10^6$ of cultured cells were resuspended in 500 μl of buffer A. The samples were incubated on ice 15 min. NP-40 was then added to a final concentration of 0.5% and the samples were lysed by vortexing for 10 s. at 4 °C. The samples were centrifuged for 5 min at 500 g_{max} to recover the nuclei which were resuspended in 150 μl buffer C (20mM Hepes pH7.9; 1.5mM $MgCl_2$; 420mM KCl; 0.2mM EDTA; 25% Glycerol; 100ng/ml Aprotin; 5ng/ml Leupeptin; 0.5mM PMFS) and incubated on a shaker at 4 °C for 30 min. After 20 min centrifugation at 14,000 g_{max} the supernatant, which contained the nuclear extract, was collected in a new eppendorf tube. The nuclear extract was stored at -80 °C. EMSA probes were synthesized by PCR, using as a template the wild-type or mutated reporter constructs of the *Mrps12-Sarsm* intergenic region, plus primers as indicated in the Supplementary Tables in the articles (I, II, III). In each case the identity of the product was verified by sequencing before proceeding. After dephosphorylation with calf intestinal alkaline phosphatase (Fermentas) under manufacturer's recommended conditions, 0.6 ng of probe DNA was labelled using 8 U of T4 Polynucleotide Kinase (Fermentas) in a 15 μl reaction containing 10 μCi of [γ-^{32}P]ATP (Amersham, 6000 Ci/mmol) for 1 h at 37 °C. The volume was adjusted to 100 μl by the addition of water, and 20 μl EMSA reactions were set up in binding buffer (25 mM HEPES-KOH, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Tween-20, 2 mM DTT, 500 mM KCl, pH 7.9) using 1 μl of probe, 5 μg of nuclear extract (protein concentration determined using Bradford assay; Pollard HB, et al. 1978), 5 μg BSA (Fermentas) and 5 μg non-specific competitor, i.e. Poly(dI–dC)·Poly(dI–dC) or Poly(dA–dT)·Poly(dA–dT) (Amersham). Supershift reactions contained, additionally, 1 μl of the relevant antibody solution (2 mg/ml, all from Santa Cruz Biotechnology): CBF-B (G-2), i.e. NF-Y, mouse monoclonal IgG, C/EBP, rabbit polyclonal IgG, C-JUN, rabbit polyclonal IgG. Supershift reactions were preincubated for 20 min at room temperature prior to addition of the probe. After incubation at room temperature for 30 min reactions were analysed on 5% polyacrylamide gels run at 4 °C in TBE buffer at 6 V/cm for 1 h, followed by 10 V/cm for a further 3–5 h, depending on the fragment size, before exposure to X-ray film.

4.9 *In vivo* footprinting

NIH 3T3 cells were grown to 80% confluence. After washing with serum-free medium, cells were treated for 6 min at room temperature with freshly prepared serum-free medium containing 0.2% (v/v) DMS. The cells were rapidly washed in cold Hank's Balanced Salt Solution (HBSS; Gibco-BRL), detached by trypsinization, and resuspended in 10 ml buffer A (Buffer A: 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris-HCl pH 8.0, 0.5 mM spermidine, 0.15 mM spermine, and 2 mM EDTA), then one volume of buffer A (10 ml) containing 1% of NP-40 was then added to the samples which were incubated on ice for 5 min and centrifuged at 5000 g_{max} for 10 min at 4 °C to sediment the nuclei, which was then washed in 10 ml of buffer A. and spun at 5000 g_{max} for 10 min at 4 °C. The nuclei were resuspended in 2 ml of buffer B (150 mM NaCl and 5 mM EDTA pH 7.8) and an equivalent volume of buffer C (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 20 mM EDTA, and 1% sodium dodecyl sulfate) was added together with proteinase K to a final concentration of 450 μg/ml. Following 3 hours of incubation at 37 °C, RNase A was added to a final concentration of 150 μg/ml, and the samples were incubated an additional hour at 37 °C. The DNA was purified by successive extraction with 1 volume of phenol, 1 volume of phenol:chloroform, 1 volume of chloroform and finally resuspended in HE buffer (10 mM HEPES pH 7.4, 1 mM EDTA) at a concentration of around 100 μg/ml. For the chemical cleavage reaction I used 50 μg of DNA fully dissolved in 50 μl of H_2O. Control DNA (50 μg) was methylated *in vitro* according to the Maxam and Gilbert reaction: briefly 50 μg of DNA was dissolved in 5 μl of H_2O plus 200 μl of DMS buffer (50 mM sodium cacodylate and 1 mM EDTA pH 8.0) and 1 μl of DMS. The three samples were properly mixed and incubated at 20 °C for 30, 45 or 60 s. Reactions were stopped by adding 50 μl of DMS stop buffer (1.5 M sodium acetate pH 7.0 and 1 M β-mercaptoethanol) and the DNA was precipitated with 750 μl of precooled absolute ethanol. Following 15 min incubation on ice the samples were centrifuged for 15 min at 15,000 g_{max} at 4 °C. The supernatant was removed and the samples were spun in order to eliminate the supernatant. The DNA pellet was dissolved in 405 µl of H_2O and re-precipitated with 45 µl of 3 M sodium acetate pH 7 plus 1 ml of precooled ethanol with a 15 min incubation on ice. After centrifugation the DNA was washed with precooled 80% ethanol and resuspended in 50 µl of H_2O. 50 µl of freshly prepared 2M piperidine was added to the DNA samples (both *in vivo*- and *in vitro*-methylated), and the samples were incubated at 82 °C for 30 min. The three *in vitro* methylated samples were mixed together and the volume of all the samples was brought to 405 µl with H_2O. The cleaved DNA was then precipitated by adding 10 µl of 3 M sodium acetate (pH 5.2), 2 µl of glycogen (20 µg/µl), and 1 ml of precooled absolute ethanol. Following 15 min incubation on ice, the samples were centrifuged for 15 min at 15,000 g_{max} at 4 °C. The DNA pellet was washed twice with precooled 80% ethanol and dissolved in H_2O at a concentration of 0.4 µg/µl. The size of the fragments was verified to be in the range 100–500 bp by 1.5% alkaline agarose gel electrophoresis.

4.10 Ligation-mediated PCR

Primer extension was performed using 2 µg of DNA, 3 µl of 10 X Pfu buffer (10X Pfu buffer: 200 mM Tris-HCl, pH 8.8, 20 mM $MgSO_4$, 100 mM NaCl, 100 mM $(NH_4)_2SO_4$, 1% (v/v) Triton X-100, 1 mg/ml nuclease-free BSA) and 1 pmol of primer 1 in a total volum of 25 µl. The PCR was run under these conditions: 98 °C for 3 min, 45-50 °C, depending on the T_m of primer 1 for 20 min, and then to 4 °C. Following the addition of 5 µl Pfu mix (Pfu mix buffer: 1.5 mM of each dNTP and 1.5 U cloned *Pfu* DNA polymerase, also named *Pfu* exo$^-$ 2.5 U/µl, Stratagene) to the samples, the temperature was brought back to 45-50 °C for 30 seconds, increased to 75 °C for 10 min at a rate of 0.3 °C/s and the samples were then cooled to 4 °C. The primer extension products were mixed with 45 µl of ligation mix (1.35 µl of 1 M DTT, 0.5 µl of 100 mM ATP, 0.15 µl of 5 µg/µl BSA, 1.1 µl of Tris-HCl, pH 7.4, 5.0 µl of 20 pmol/µl linker, 3.25 µl of 1 U/µl T4 ligase, and 33.65 µl of H_2O) and after an overnight incubation at 18 °C the DNA was precipitated with 28.75 µl of 7.5 M ammonium acetate, 0.25 µl of 0.5 M EDTA (pH 8), 2 µl of glycogen (20 µg/ µl), 275 µl of precooled absolute ethanol and incubated on ice for 30 min. After centrifugation and washing with precooled 80% ethanol the DNA was resuspended in 50 µl of H_2O. The PCR amplification was performed by adding 50 µl of

Pfu DNA polymerase mix (2X cloned *Pfu* buffer, 0.5 mM of each dNTP, 10 pmol of LP25 -Linker Primer, 10 pmol of primer 2, and 3.5 U of cloned *Pfu* DNA polymerase) to the samples and PCR amplification was performed using the conditions described in the table below (Table 4.1). Once the PCR amplification step was completed 5 µl of labeling mix (lx Pfu buffer with 2 mM each dNTP, 2.3 pmol of gene-specific primer 3) was added the PCR labeling was run under the following conditions: 3.5 min at 98 °C, 2 min at 69 °C, 10 min at 76 °C, 1 min at 95 °C, 2 min at 69 °C, and 10 min at 75 °C. The reaction was stopped by placing the samples on ice and adding 25 µl of stop solution (20 mM EDTA 1.56 M sodium acetate, pH 5.2). The PCR product was then ethanol precipitated and resuspended in 14 µl of of premixed formamide loading dye (94% formamide, 2 mM EDTA, pH 7.7, 0.05% xylene cyanol FF, and 0.05% bromophenol blue). The sample was denatured at 94 °C for 5 min and 7 µl was loaded onto a 6% polyacrylamide TBE sequencing gel.
For *In vivo* footprinting from tissues, mouse tissue, minced in PBS buffer and properly cleaned from blood, was treated for 6 min at room temperature with freshly prepared serum-free medium containing 0.2% (v/v) DMS. The reaction was stopped by adding 10 ml of of PBS, containing 0.4 M β-mercaptoethanol. Tissue was washed in PBS and trypsinized to isolate the cells. The DNA was then isolated as previously described.

Addition of linker was carried out: in aliquots of 500 µl, annealing in 250 m*M* Tris-HCl, pH 7.7, 20 pmol/µl each 25-mer and 11-mer oligonucleotide, heating at 95 °C for 3 min, transferring quickly at 70 °C, and cooling gradually to 4 °C over a period of 3 h. Linkers were stored at –20 °C and thawed on ice before use. Linker sequences were: L25 (60 pmol/µl, 5′-GCGGTGACCCGGGAGATCTGAATTC-3′), L11 (60 pmol/µl, 5′-GAATTCAGATC-3′), 2 *M* Tris-HCl, pH 7.7 and 1 M $MgCl_2$.
From primer labeling: Oligonucleotide (50 picomol) was incubated at 37 °C for 30 min in 10X T4 Polynucleotide Kinase forward buffer 5 µl, T4 kinase 2 ul (20units), fresh 32 Phosphorus- [γ-^{32}P]ATP 5 µl and H_2O to 80 µl, and then purified with mini quick spin oligo columns-Roche.

Electrophoresis was carried out on 8% polyacrylamide gel polymerized with 1 ml of 10% ammonium persulfate (APS) and 30 μl of *N*,*N*,*N*',*N*'-tetra-methylethylenediamide (TEMED)

Table 4.1.
Exponential Amplification Steps Using Cloned Pfu DNA Polymerase for Ligation-mediated PCR

	Denaturation condition T in °C for D in s)	Annealing condition (*T* is the T_m of the oligonucleotide for D in s)	Polymerization Time (D in s) *T* is the same for all cycles: 75°C for *Pfu* and 74°C for *Taq*
Cycle	*Pfu*	*Pfu*	
1	98 for 300	T_m for 180	180
2	98 for 120	T_m –1°C for 150	180
3	98 for 60	T_m –2°C for 120	180
4	98 for 30	T_m –3°C for 120	180
5	98 for 20	T_m –4°C for 90	150
Repeat cycle 5, 13 more times (add 5 s per cycle for annealing and polymerization)			
19	98 for 20	T_m –3°C for 240	240
20	98 for 20	T_m –2°C for 240	240
21	98 for 20	T_m –1°C for 240	240
22	98 for 20	T_m for 240	600

4.11 Chromatin Immunoprecipitation (ChIP) assay

NIH 3T3 cells or HEK293 were grown to 80–90% confluence in 10 cm culture plates. After cross-linking for 10 min with 1% formaldehyde in serum-free medium, phosphate-glycine buffer was added to a final concentration of 0.125 M and cells were washed twice with ice-cold phosphate-buffered saline (PBS). Nuclei were isolated as for EMSA and lysed essentially as described in (Spencer VA, et al. 2003), except that an SDS concentration of 0.1% instead of 1% was used. The chromatin lysate was sonicated on ice to an average DNA length of 600 bp. Chromatin was pre-cleared with blocked Sepharose A and ChIP assays were performed as described by (Spencer VA, *et al.*2003) using 8 µg of either the anti-NF-Y mouse monoclonal antibody described above or, as negative control, M2 anti-FLAG® mouse monoclonal (Stratagene). The final PCR step used primers 5′-GGAGTGGAAACAAGAAGTCACTCAT-3′

and 5-′CTGAGTAGGCCCCCAAGGACC-3′ for mouse, HsarsmF_1 and RPMS12F_5 for human (see Suppl. table in article II). PCR reaction products were analysed on a 1.5% agarose-TBE gel stained with ethidium bromide and visualized under ultraviolet (UV) light.

5. Results

5.1 Molecular architecture of the Mrps12-Sarsm bidirectional promoter (I, II)

The genes for mitoribosomal protein S12 (*Mrps12*) and mitochondrial seryl-tRNA synthetase (*Sarsm* or *Sars2*) are oppositely transcribed from a conserved promoter region of <200 bp in both humans and mice. The two genes are located in chromosome 19 in humans and chromosome 7 in mice. The intergenic region (in between the two start codons) is around 500 bp long. Comparison of the human and mouse bidirectional promoter revealed a highly preserved overall organization. EST data revealed that the 5' UTR of *Sarsm* was extremely short (26 nt) and largely identical in mouse and human. The non-transcribed portion of the intergenic sequence is 184 bp in mice and only 7 bp longer in humans. The sequence closer to *Sarsm* is dissimilar between the two species, except for the presence of two CCAAT box sequences which present similar spacing. The rest of the promoter region presents around 70 % homology between human and mouse. For both species this region contains two additional CCAAT box sequences identically spaced, and a putative NRF-2 binding site. Two start site clusters for *Mrps12* are found in virtually identical positions in both species. The first overlaps (human) or immediately follows (mouse) the putative NRF-2-binding site. A second cluster of *Mrps12* transcriptional start sites coincides approximately with the start of *Mrps12* the intron (See Figure 5.1. Molecular architecture of the *Mrps12-Sarsm* bidirectional promoter).

5.2 Reporter analysis of the Sarsm-Mrps12 intergenic region (I, II)

The functional studies of the promoter were performed using a dual luciferase reporter vector, in which the transcriptional activities in the two opposite directions could be tested simultaneously via the readouts of firefly and renilla luciferase. I commenced by inserting in both orientations the full intergenic region, 480 bp in mouse and 510 bp in human, into the luciferase reporter vector pFRL (See Figure 5.2)

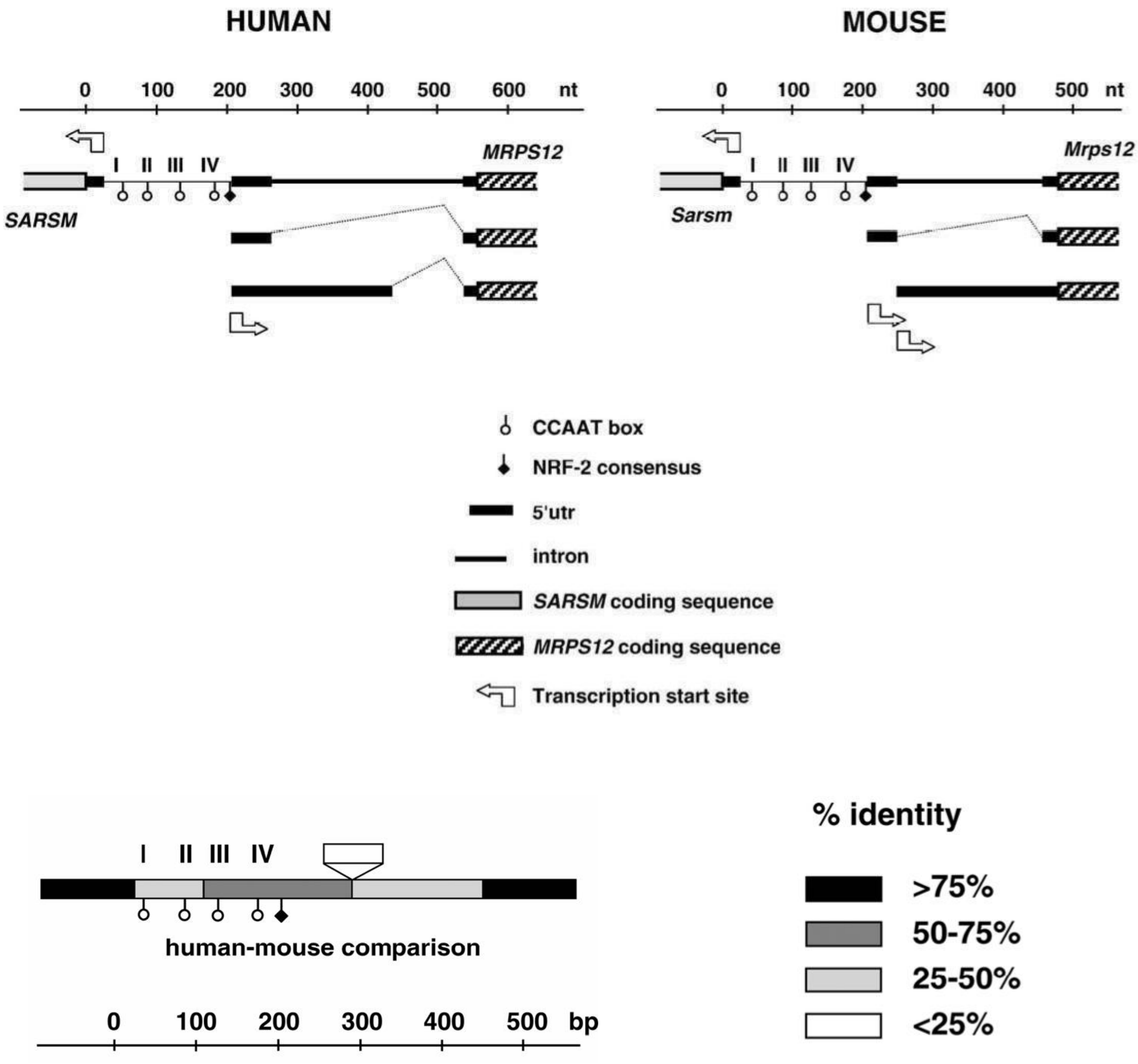


Figure 5.1. Molecular architecture of the *Mrps12-Sarsm* bidirectional promoter region found in humans and mice. For complete sequence of the mouse promoter see Suppl. Figure of (I): for complete sequence of the human promoter see Suppl. Figure (II). Numbering commences at the nucleotide pair immediately 5′ to the *Sarsm* start codon. Based on EST data, mouse and human *Sarsm* (*SARSM*) mRNAs each exist in a single isoform. Mouse *Mrps12* mRNA has at least two major splice variants as shown, with different transcription starts. In human, *MRPS12* mRNA has a single transcription start site but again two splice variants.

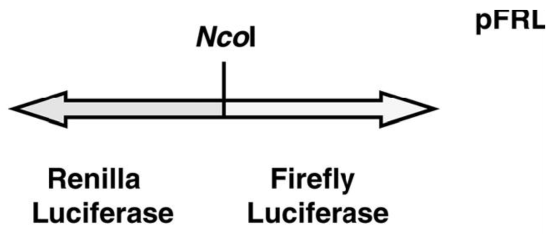


Figure 5.2. Reporter analysis of the *Mrps12-Sarsm* bidirectional promoter. The promoter region (from 1 to 480 nt or to 510 nt depending on the species, e.g. immediately flanked by the two start codons) was cloned into the *NcoI* site of the customized vector pFRL in both orientations.

5.3 Mutational analyses: mouse (I)

I performed a series of site-directed mutageneses in order to destroy putative binding sites in the mouse promoter that were predicted to interact with common transcription factors. I then evaluated the changes in promoter activity using the dual luciferase reporter vector (Figure 5.3). I started by destroying the CCAAT boxes. I did this by replacing the central CA of each CCAAT box, both individually and in all combinations, by TG. Destroying either CCAAT box I or IV resulted in a change of directionality but not activity, whereas loss of CCAAT boxes II and/or III caused only a modest drop in the total amount of expression. Destroying CCAAT box I alone or in combination with CCAAT boxes II or III, resulted in a large relative activity increase in favour of *Sarsm*, while loss of CCAAT box IV alone, had the opposite effect. Destroying all four CCAAT boxes, dramatically abolished the promoter activity, with less than 15% activity left for *Sarsm* or *Mrps12*, thus indicating a crucial role of the CCAAT boxes in promoter activity. CCAAT box I alone could maintain half of total transcription, mainly in favour of *Mrps12*, which was enhanced in the presence also of CCAAT box II. Deletion of CCAAT box IV alone produced an increase in total transcription, mostly enhancing *Sarsm* expression. The additional presence of CCAAT boxes II and/or III did not affect directionality while it reduced the activity increase. Leaving intact only CCAAT box II or

III, transcription was still supported at 30-40% of the wild-type control. Based on sequence occupancy observed by *in vivo* footprinting, I also mutated some other potential binding sites involved in transcription, such as for NRF-2 and AP-1 (which is located between the 5′ UTR of *Sarsm* and the CCAAT box I and between the CCAAT boxes III and IV). Destruction of the putative NRF-2 binding site reduced promoter activity to about 50% in each direction. Destroying the AP-1 binding site in either or both of the two regions where protected and hyperaccessible sites were adjacent resulted in a 30% drop on transcription, again without changing directionality. Among the many mutations that I introduced to destroy potential binding sites, based on bioinformatic predictions, I found little evidence for functional binding sites. A cluster of mutations (construct Mu3) introduced within the end of the phylogenetically conserved core of the *Mrps12* first intron, up regulated promoter activity, similarly as seen for an adjacent deletion site (M2) (I). Mu3 increased the total activity 3 fold. Based on results from Mu3 in combination with the CCAAT box mutations, it was suggested that the Mu3 element causes transcriptional repression in combination with the CCAAT box array, most potently together with CCAAT box IV. However, I could not detect any protein-binding to this region by EMSA (see section 5.5. below), nor by *in vivo* footprinting. The putative binding sites present in the Mu3 region, based on bioinformatic prediction, are for NF-kB and ELK.

5.4 Mutational analyses: human (II, III)

The human bidirectional *MRPS12/SARSM* promoter contains a similar array of four CCAAT box elements (Fig. 5.1), with the same orientation, and approximately the same positions, as in the homologous mouse promoter. Thus, I tested whether the CCAAT boxes regulate transcriptional activity in the human promoter similarly as seen for the mouse promoter. Each CCAAT box was destroyed systematically, in all possible combinations (Figure 5.4). Surprisingly, and in contrast to the results obtained with the mouse promoter, elimination of all four CCAAT boxes (construct box-0) still gave substantial transcriptional activity in both directions.

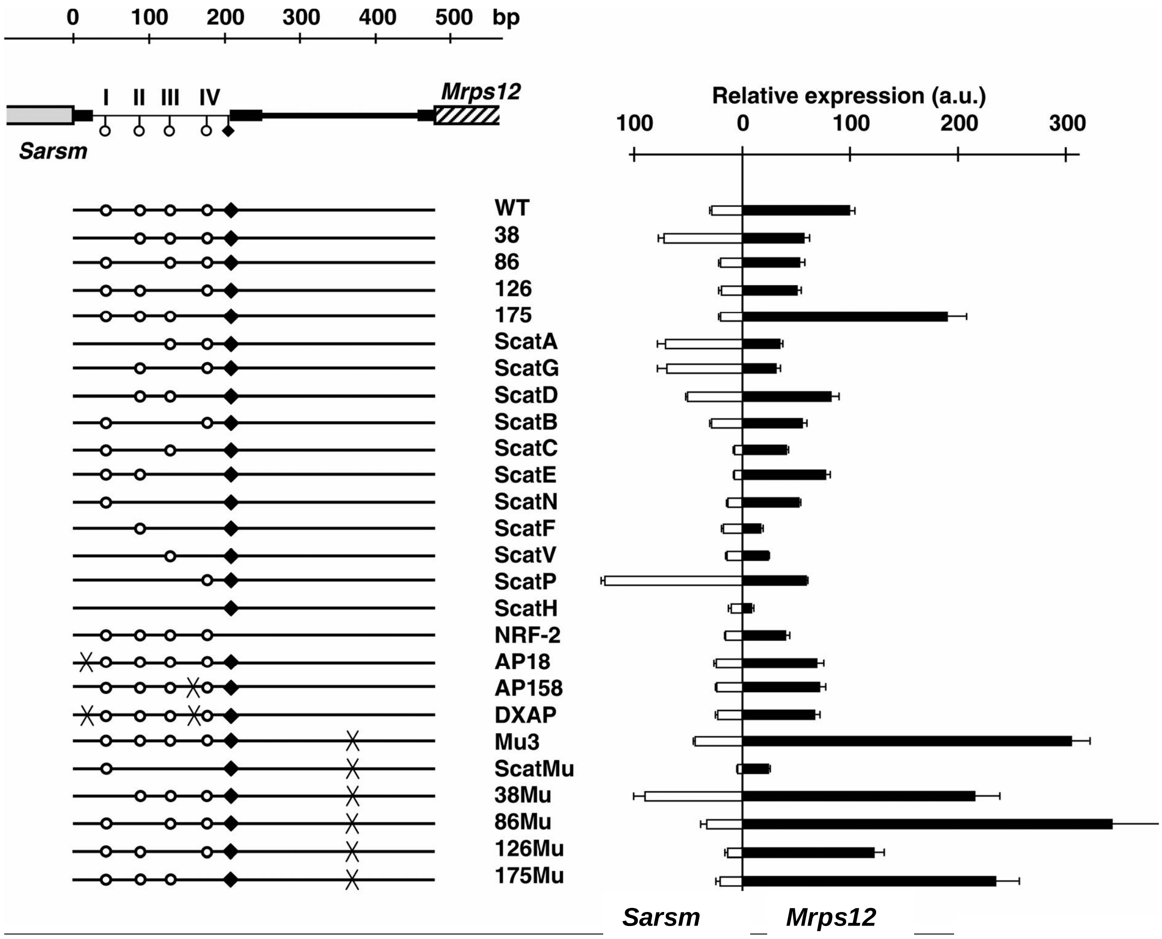


Figure 5.3. Reporter analysis of mutated versions of the *Mrps12-Sarsm* bidirectional promoter. The intact promoter elements remaining in each reporter construct are indicated by open circles (CCAAT boxes) and filled diamonds (NRF-2 site), aligned with the schematic map. The locations of other, specific sets of point mutations introduced at other sites are shown by crosses. For details of mutations and all constructions see Supplementary Table 1 and Supplementary Figure 9b (I).

Destruction of some combinations of CCAAT boxes gave substantial shifts in the relative amounts of transcription in the two directions, and some also showed substantial increases in overall promoter activity. The data indicate that the CCAAT boxes acted in combination, although not in a simple manner. Destruction of CCAAT boxes II and/or III had little effect. Destruction of CCAAT boxes I plus IV also had a small effect, although the directionality of transcription was clearly shifted in favor of *MRPS12*. Elimination of CCAAT boxes I plus II led to a strong shift in favor of the *SARSM* direction, regardless of whether CCAAT box IV was still present. Finally, a construct retaining only CCAAT box I or IV showed a shift towards the *SARSM* direction, whereas the retention of only CCAAT box II or III favored transcription in the opposing direction.

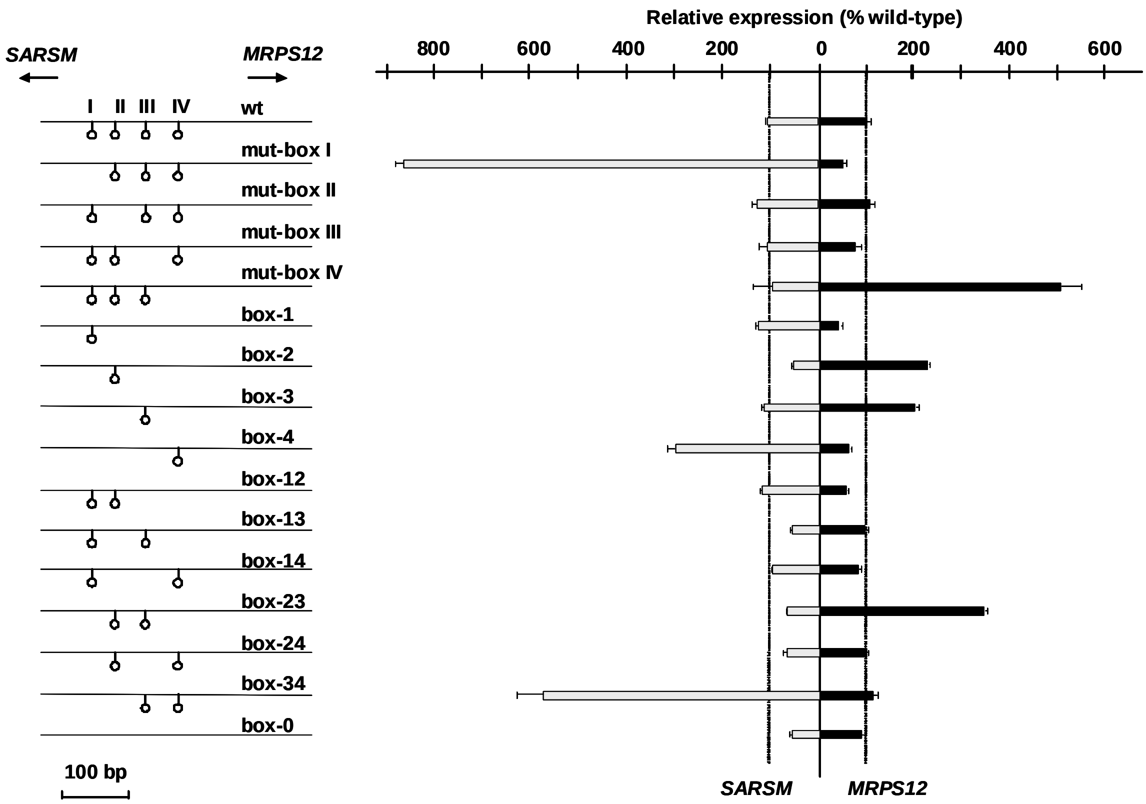


Fig. 5.4. Reporter analysis of the human *MRPS12/SARSM* bidirectional promoter in HEK293 cells. The promoter region (from nucleotide 1 to 557 as numbered in Supplementary Fig.1 of (III), i.e. immediately flanked by the two start codons) was cloned into the *NcoI* site of the customized vector pFRL (I). Point mutations in which each of the four CCAAT boxes (open circles) or the NRF-2 consensus binding sequence (filled diamond) were individually destroyed were also tested, as shown. Expression in the two directions was calculated based on luciferase activities after transfection, as follows. First, activities were normalized to that of β-galactosidase in the same extracts, to correct for transfection efficiency.

5.5 EMSA of mouse promoter (I, II, III)

Following the identification of *cis*-acting elements within the mouse bidirectional *Mrps12/Sarsm* promoter by luciferase reporter assays, I then studied protein binding to such sites by means of EMSA. The probes were synthesized by amplifying specific regions of the promoter and were reacted with nuclear extract from NIH 3T3 cells, or from mouse organs such as brain and liver. The intergenic region was initially divided into four overlapping fragments of around 120 bp each (Figure 5.5.A). Three of the four overlapping fragments gave clear evidence of specific protein-binding complexes. The specific complexes formed by fragments A and B (Figure 5.5.C), each of which contains two of the CCAAT boxes, were reciprocally competed, indicating that they most likely involved the same protein(s) (sup. figure in I). Fragment C gave one clear retarded band which was competed only by fragment C itself. Fragment D gave no signal indicative of sequence-specific protein-binding (I). The CCAAT box containing fragments A and B showed a complex pattern of oligonucleotide competition (I). Antibody supershift experiments demonstrated that the factor binding to the CCAAT box array was NF-Y (Figure 5.5.B and 5.5.C). An antibody against NF-YA subunit supershifted the complex formed by the fragment containing all four CCAAT boxes, fragment E1234 (Figure 5.5. B), or the one formed by fragments A and B containing two CCAAT boxes each (Figure 5.5.C) or by any of the CCAAT boxes I, III or IV individually (I). An antibody against c-Jun did not produce supershift (Figure 5.5.B), although the results from the luciferase assay and *in vivo* footprinting indicated the presence of possible protein-binding at the two AP-1 binding sites. Less consistently, an antibody against C/EBP was able to produce a supershift. However, the signals were clearly seen only for large fragments containing CCAAT boxes III and IV. A clearer supershift with anti-C/EBP was observed when CCAAT box IV was destroyed, i.e. probe E123 (Figure 5.5.B). The band which was produced by supershift with the antibody against C/EBP appeared to migrate in the same position as the one which was generated by the antibody against NF-Y. I tentatively conclude that, *in vitro*, C/EBP interacts weakly or transiently with this complex. Using nuclear extract from mouse liver I was able to obtain a similar pattern of protein-binding to fragments containing the CCAAT boxes (Figure 5.5.B). Once again, a clear supershift was obtained using an antibody against NF-Y. The results of EMSA experiments can be summarized as follows: sequence-specific protein-binding was detected to the CCAAT box array and to a sequence element within the *Mrps12* first intron. The protein-binding to the CCAAT box array *in vitro* was identified as NF-Y. At least in nuclear extracts from some tissues, C/EBP also appeared to be present within the same complex.

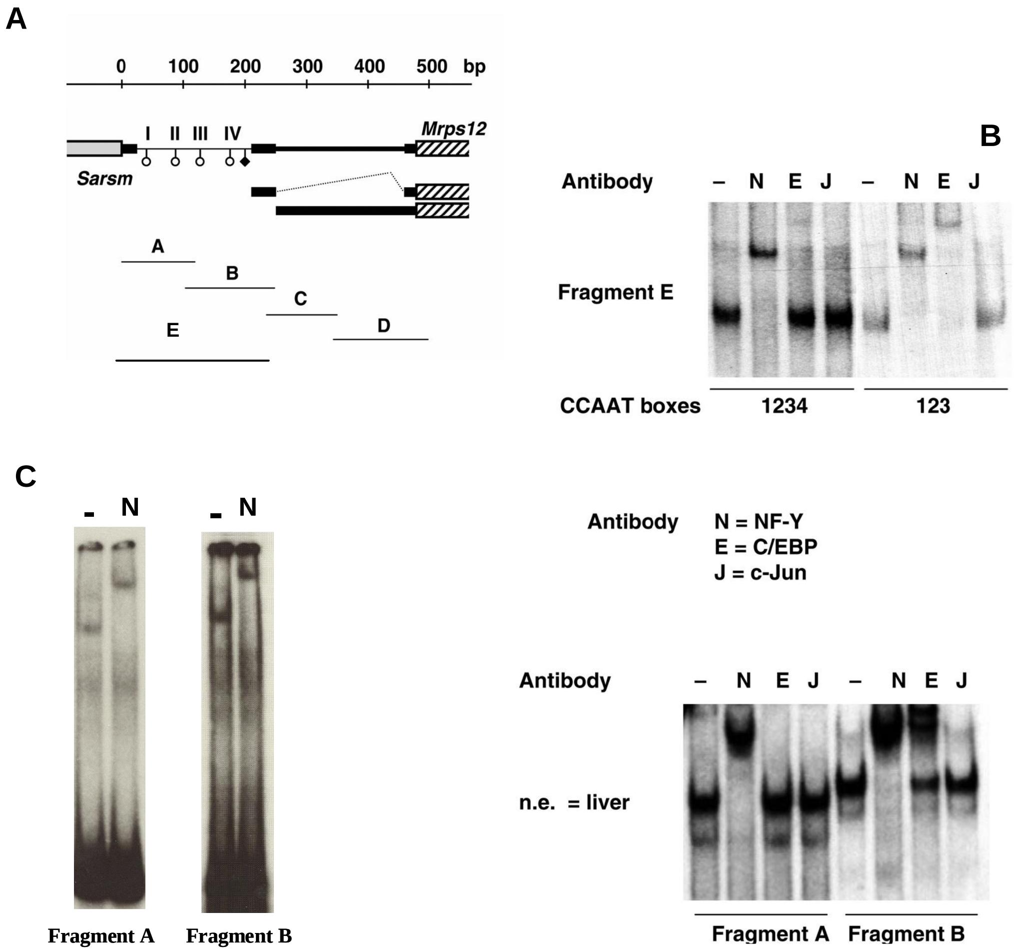


Figure 5.5. EMSA analysis of protein-binding to CCAAT box array in mouse. Figure 5.5.A: Schematic map showing locations of the four CCAAT boxes and probe fragments used. EMSA using wild-type and mutant versions of fragments A (from nt 1 to 125), B (from nt 120 to 250) and E (from nt 1 to 250), created by PCR from mutated reporter constructs. Figure 5.5.B: Antibody supershift EMSA using wild-type and mutated fragment E, with intact CCAAT boxes as denoted by Arabic numerals, and antibodies as indicated, nuclear extract from NIH 3T3 cells. Antibody supershift EMSA using nuclear extract from mouse liver, probe fragments and antibodies as indicated. Figure 5.5.C: EMSA using as probes fragment A and B with (N) and without (-) antibody supershift.

5.6 EMSA of human promoter (II, III)

EMSA experiments, using overlapping fragments from the human promoter and *Mrps12* first intron (Figure. 5.6), identified a complex which was formed by fragments containing CCAAT boxes. A ten-fold mass excess of other CCAAT box containing fragments competed efficiently, except for a fragment containing only CCAAT box III, which was a comparatively poor competitor. An antibody against NF-Y supershifted the CCAAT box containing complexes, but antibodies against C/EBP (or c-Jun, not shown) failed to bring about a convincing supershift. A probe fragment spanning the *Mrps12* 5′ UTR and first intron gave no EMSA signal (Figure 5.6).

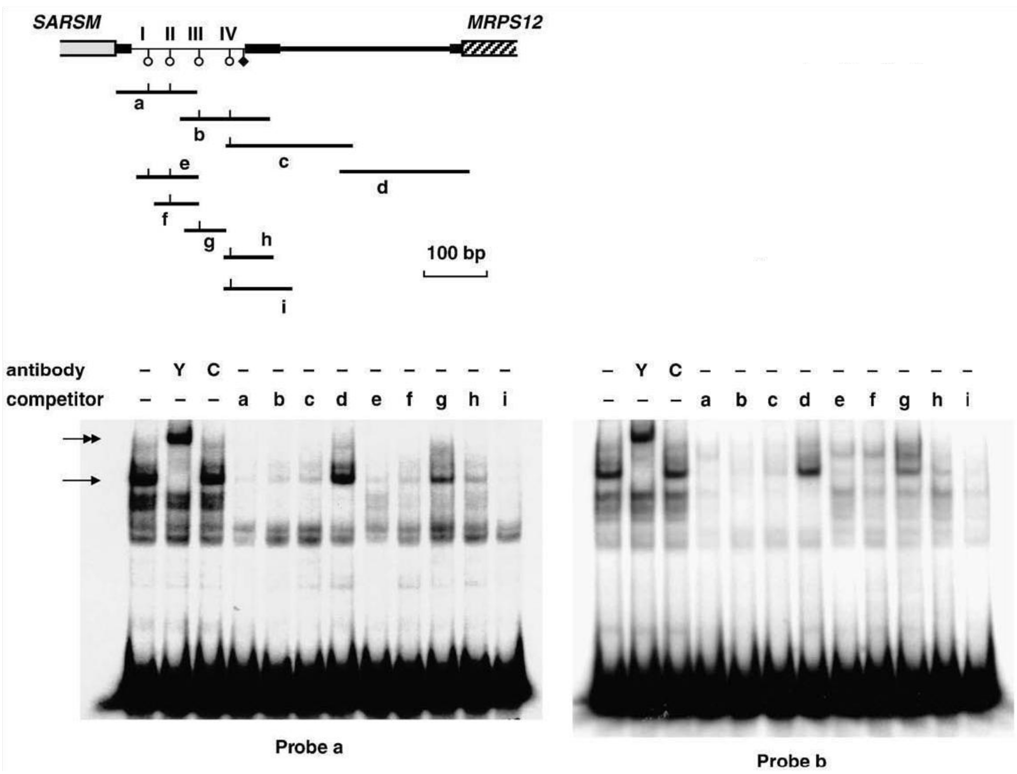


Figure 5. 6. EMSA analysis of protein-binding to the human bidirectional promoter region. The main features of the promoter region (top left) are reproduced from Fig. 5.1, aligned with the nine fragments tested in the assays, denoted a through i. EMSA gels for probes a and b are shown. Reactions contained a 10-fold molar excess of competitor fragments as indicated. Supershift experiments used antibodies against NF-Y (Y) and C/EBP (C). An antibody against C-Jun was tested in separate supershift experiment (not shown) and found to be negative. The arrow indicates the major protein–DNA complex inferred to represent binding at the CCAAT boxes. The double-headed arrow denotes the complex supershifted by the antibody against NF-Y.

5.7 CCAAT box orientation influences activity but not directionality of the *Mrps12/Sarsm* promoter (III)

Based on the observation that the CCAAT boxes appear to be required to maintain the correct ratio of transcription in the two directions, I tested whether their orientation influences the directionality of transcription. To do this I inverted the 15 bp in which each CCAAT box is embedded, then measured the effects on transcription using the dual reporter system (Figure. 5.7). Inverting any of the CCAAT boxes of the human promoter (Figure 5.7.A) had virtually no effect on the directionality of transcription, although inversions of CCAAT box I or CCAAT box III, or substitution of CCAAT box I by a copy of the 15 bp of CCAAT box IV, led to a drop in promoter activity in *both* directions. A more limited inversion of just the core CCAAT element of CCAAT box I also had only a slight effect. Similar inversion and/or exchange of CCAAT boxes in the mouse promoter again produced virtually no change in directional selectivity (Figure 5.7.B). Inversion of any of the CCAAT boxes individually caused a decrease in transcription, with inversion of CCAAT box III having the strongest effect (Figure 5.7.B), as in the human promoter (Figure 5.7A). Replacement of CCAAT box I by CCAAT box III or IV (Fig. 7B, constructs 4234 and 3234) caused a decrease in transcriptional activity, whereas replacement of CCAAT box IV by CCAAT box I (construct 1231) had virtually no effect. Replacement of the weakly NF-Y binding CCAAT box III with the stronger binding CCAAT box I (construct 1214) resulted in increased transcriptional activity. The only cases where inversion of a CCAAT box gave increased transcriptional activity was where an inverted copy of CCAAT box IV was inserted in place of CCAAT box I (compare constructs 4234 and Inv4-234), or *vice versa* (compare constructs 1231 and 123-Inv1). The ability of NF-Y still to bind to the inverted or exchanged CCAAT boxes was confirmed by EMSA (III).

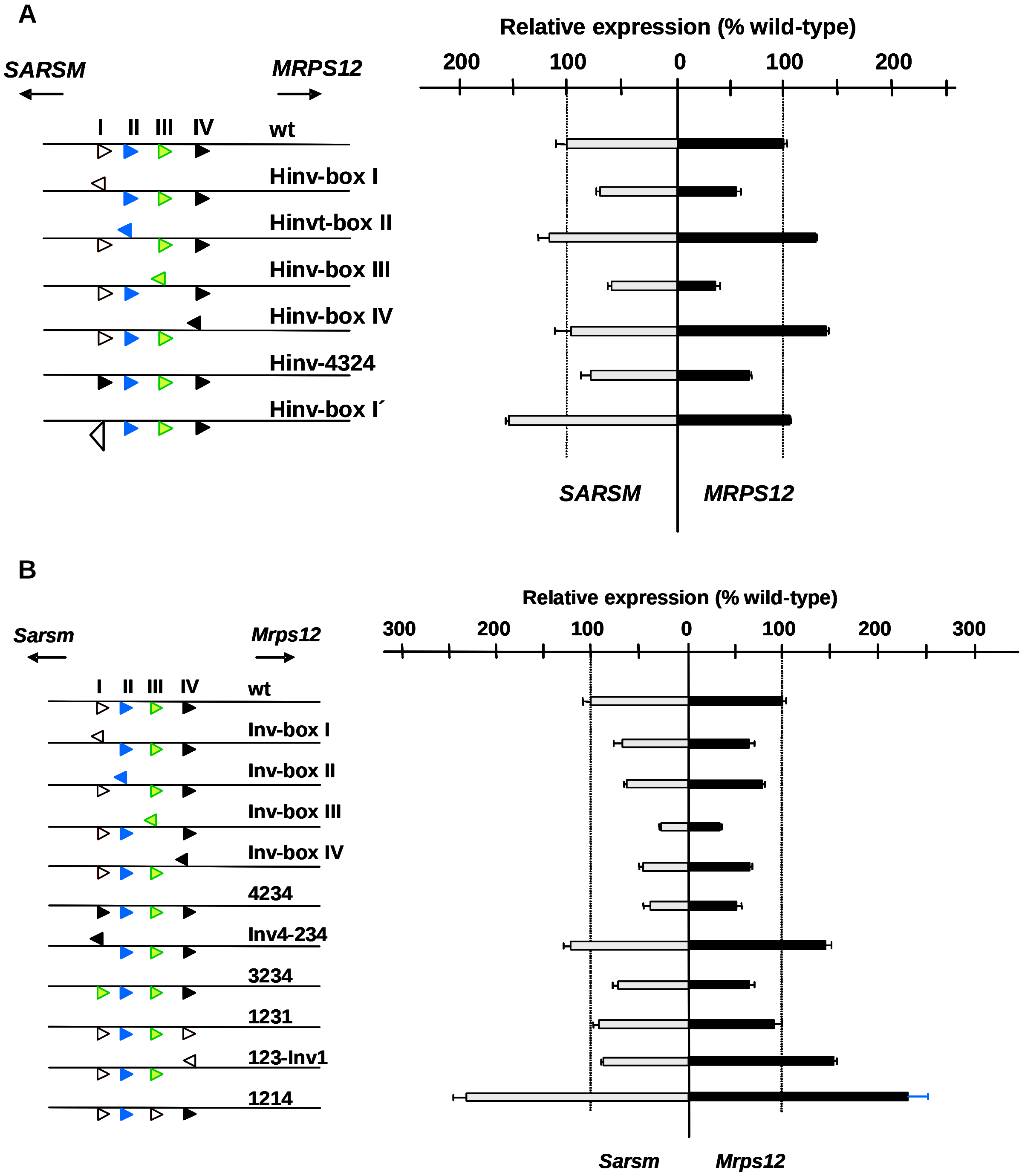


Figure 5.7: Analysis of variants of the mouse and human *Mrps12*/*Sarsm* bidirectional promoter in HEK293 cells. Figure 5.7.A: Reporter analysis of the human promoter. Constructs contain inversions or substitutions of CCAAT boxes as indicated. Construct Hinv-box1´contains a modified CCAAT box I, in which the CCAAT sequence is inverted, but not the surrounding nucleotides. Figure 5.7.B: Reporter analysis of the mouse promoter. Constructs contain inversions or substitutions of CCAAT boxes as indicated.

5.8 Promoter activity modulation by mitochondrial stress (II)

Mitochondrial stresses were found to induce a biphasic response in the *Mrps12/Sarsm* promoter in mouse cells. To address the issue of how a bidirectional promoter of two nuclear genes encoding components of the mitochondrial translational apparatus responds to mitochondrial OXPHOS stress, I treated cells with a variety of drugs acting directly or indirectly on OXPHOS, then transfected them with the bidirectional reporter constructs used previously. I then measured reporter expression after 24 h, alongside that in cells not treated with drugs (Figure 5.8.). Treatment of mouse NIH 3T3 cells (Figure 5.8.A) with OXPHOS inhibitors (antimycin plus rotenone), or with the uncoupler FCCP, produced large and comparable increases in promoter activity in both directions (3–7-fold). Inhibitors of mitochondrial protein synthesis, such as doxycycline or thiamphenicol, produced much more modest stimulation of promoter activity (1.2–1.5-fold, in several cases barely significant). When the same drugs were added back 4 h following transfection, in addition to the pre-treatment, an opposite effect resulted (Figure 5.8.B), with promoter activity in both directions greatly diminished by FCCP (to 20-30% of that untreated cells), and more modestly downregulated by the other drugs. In mouse C2C12 myoblasts, pre-treatment with the same drugs produced only the downregulation seen in 3T3 cells under more prolonged exposure (Figure 5.8.C). Downregulation was seen even at very low drug concentrations. In all cases the effects on transcription in the two directions were comparable.

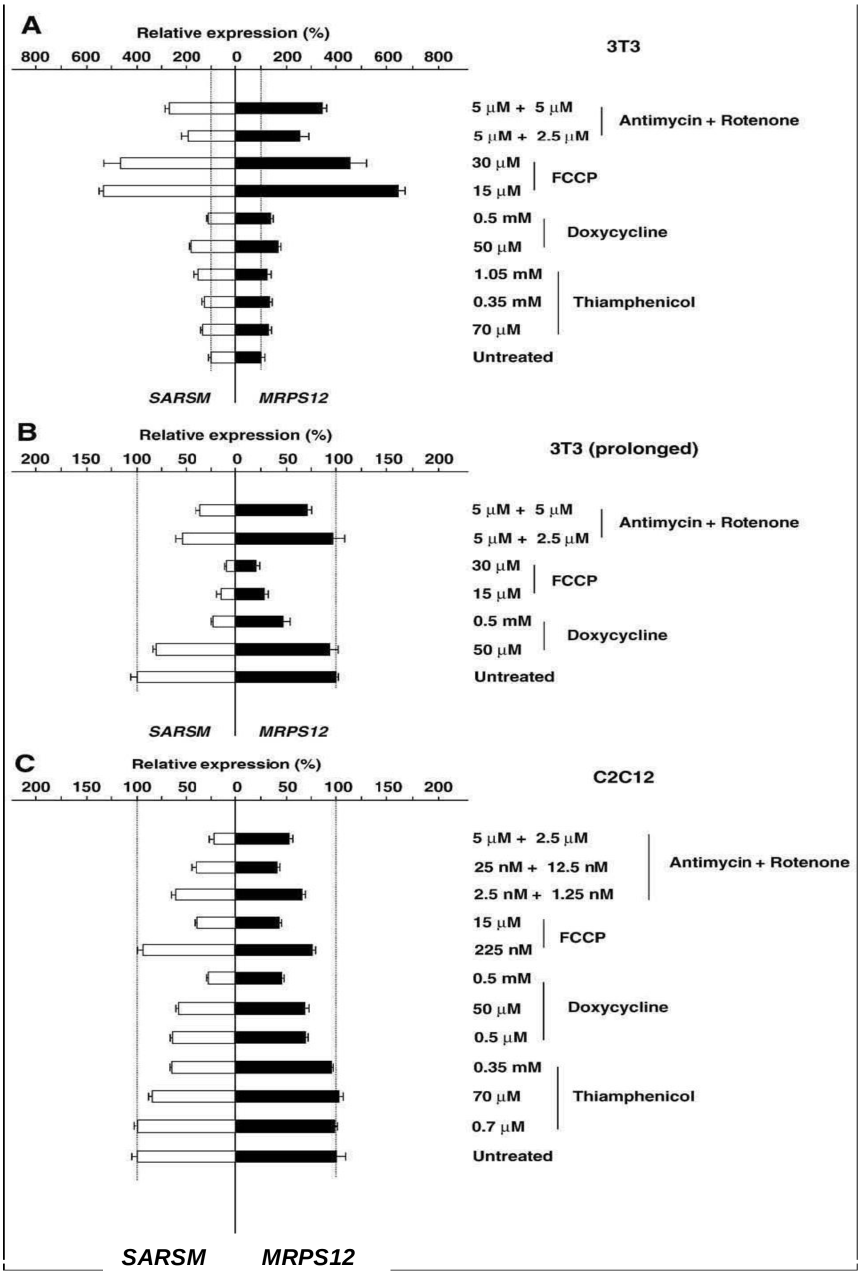


Figure 5.8. Effects of mitochondrial OXPHOS stress on transcriptional activity of the mouse *Mrps12/Sarsm* promoter. Cells as indicated were pre-treated with various drugs to inhibit mitochondrial OXPHOS or protein synthesis (A and C). In part (B; 5.8.B) 3T3 cells were also treated with the drugs after transfection (see Materials and Methods). All experiments used the wild-type dual reporter construct in which transcription in the *Sarsm* direction was assayed as firefly luciferase activity, and in the *Mrps12* direction as Renilla luciferase activity. In each case, luciferase activities were first normalized to that of β-galactosidase in the same extracts, to correct for transfection efficiency. Expression was then normalized against that supported by the wild-type promoter in the same direction, in untreated cells (taken as 100%, dotted vertical lines). Plotted data (black bars in the *Mrps12* direction, white bars in the *Sarsm* direction) are means ± SD from at least three independent transfections of drug-treated cells.

5.9 Mitochondrial stresses induce a more weakly biphasic response in the Mrps12/Sarsm promoter in human cells (II)

The results generated from looking at the cell-type response to an induced mitochondrial stress for the mouse promoter, prompted me to investigate how the human promoter responded to similar stresses in different cell types. I tested HEK293 (embryonic kidney), HeLa (cervical carcinoma) and U2OS (osteosarcoma) cells under similar conditions as the initial trials in mouse cells (12 h pre-treatment with various drug concentrations). HEK293 and U2OS cells behaved essentially the same as each other and as mouse C2C12 myoblasts: promoter activity in both directions was downregulated by all drugs tested, and even at low concentrations (II). Under prolonged drug exposure, HEK293 cells showed almost identical responses as in the case of pretreatment alone (II). In HeLa cells (Figure 5.9) the pattern was qualitatively more similar to that seen in 3T3 cells, although quantitatively less dramatic. At low concentrations of all the drugs tested there was a consistent but very modest stimulation of promoter activity in both directions (typically 1.3 to 1.5-fold), whereas at higher drug concentrations there was generally little effect, or even a slight inhibition (II). Doxycycline at high concentration (0.5 mM) produced the strongest stimulation (2-fold), although much lower doses still elicited a stimulatory effect.

5.10 Involvement of different transcription factors in regulation of the Mrps12/Sarsm promoter by mitochondrial stress (II)

I then tested for possible involvement of the CCAAT box array and other putative transcription factor binding sites by using variants of the bidirectional reporter construct carrying point mutations which destroy these various sites (Figure 5.10.A). The elimination of all 4 CCAAT boxes, even though it caused greatly reduced transcription in both directions and shifted the balance of residual transcription in the *Sarsm* direction, as shown previously (I), did not significantly affect the proportionate amount of induction by FCCP.

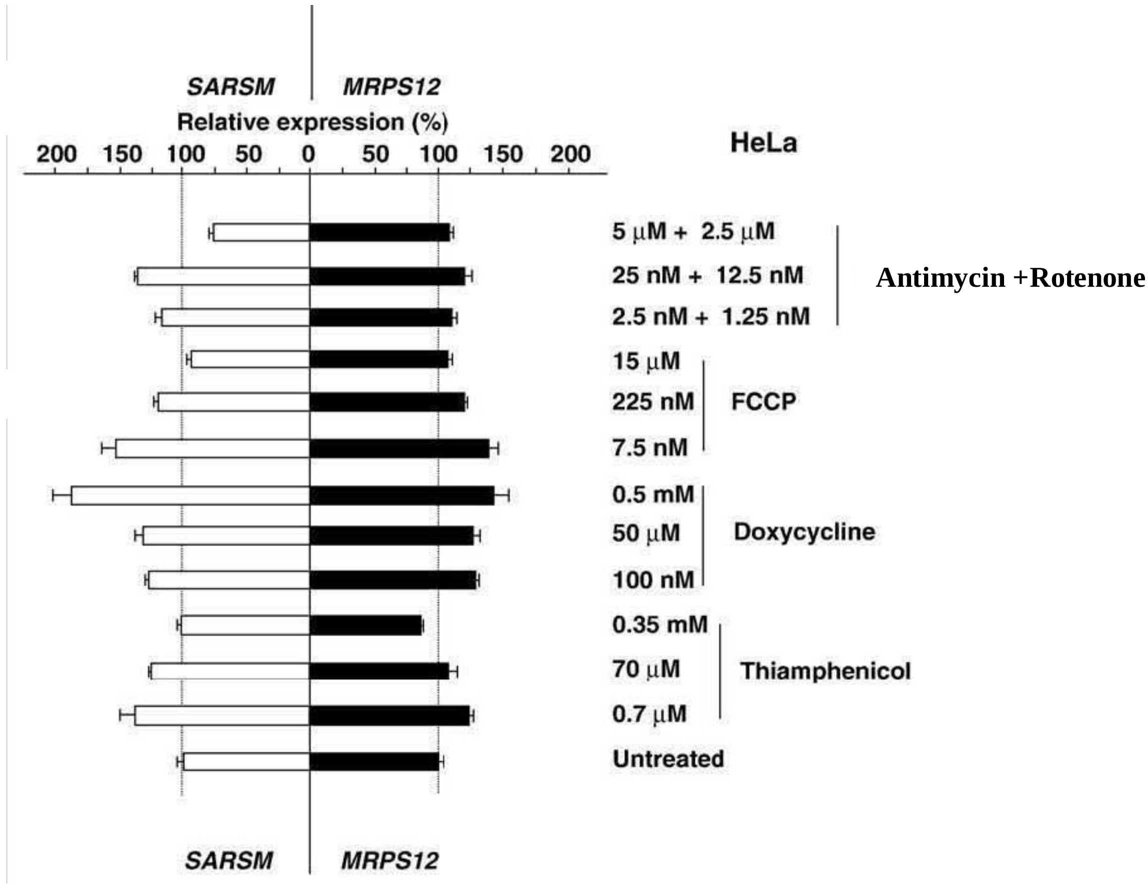
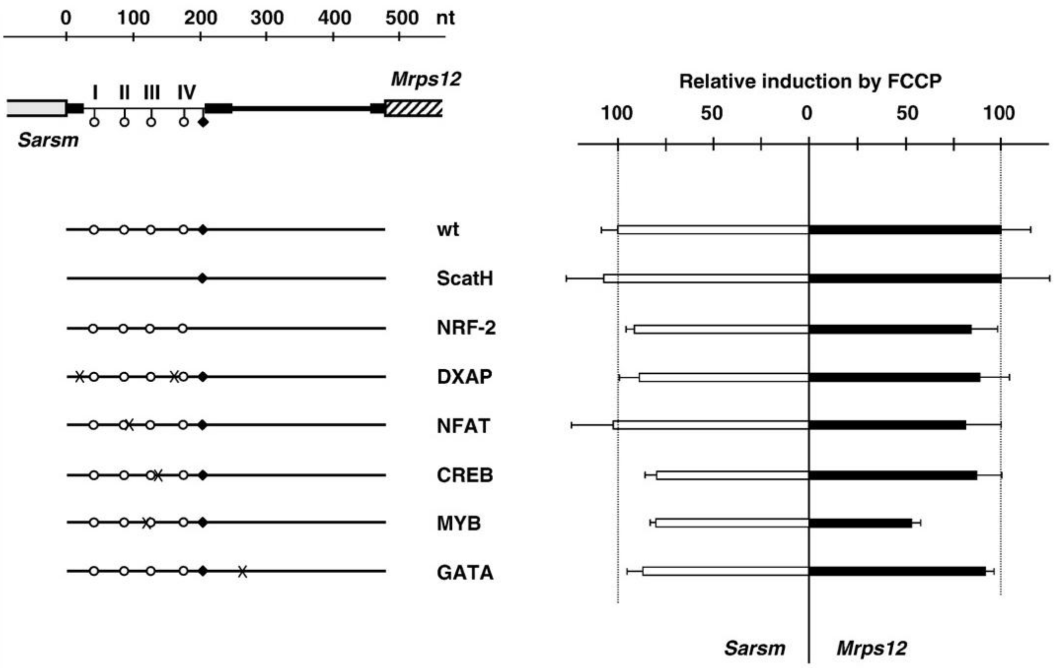


Figure 5.9. Effects of mitochondrial OXPHOS stress on transcriptional activity of the human *MRPS12/SARSM* promoter. Hela cells were treated with various drugs to inhibit mitochondrial OXPHOS or protein synthesis; see (II) for detailed description of the drugs. All experiments used the wild-type dual reporter construct in which transcription in the *Sarsm* direction was assayed as firefly luciferase activity, and in the *Mrps12* direction as Renilla luciferase activity. In each case, luciferase activities were first normalized to that of β-galactosidase in the same extracts, to correct for transfection efficiency. Expression was then normalized against that supported by the wild-type promoter in the same direction, in untreated cells (taken as 100%, dotted vertical lines). Plotted data (black bars in the MRPS12 direction, white bars in the SARSM direction) are means ± SD from at least three independent transfections of drug-treated cells.

Removal of the putative NRF-2 binding site or of the two AP-1 consensus sequences in the bidirectional promoter also did not affect induction by FCCP. Of the other putative binding sites tested none caused a substantial reduction in the proportionate induction by FCCP when eliminated, with the arguable exception of the putative binding site for MYB, which overlaps CCAAT box III, destruction of which caused a 40% reduction in transcriptional induction by FCCP in the *Mrps12* direction. In contrast, I saw a clear mitigation of the downregulation of the promoter brought about by prolonged drug exposure, when the 4 CCAAT boxes were destroyed (Figure 5.10.B). Expression in the *Mrps12* direction remained at approximately the same level as in untreated cells, and the sharp drop in expression in the *Sarsm* direction brought about by prolonged drug treatments was only half as severe as with the wild-type construct. Elimination of the NRF-2 binding consensus had a slight mitigating effect, whereas abolition of both copies of the AP-1 consensus had none.

A



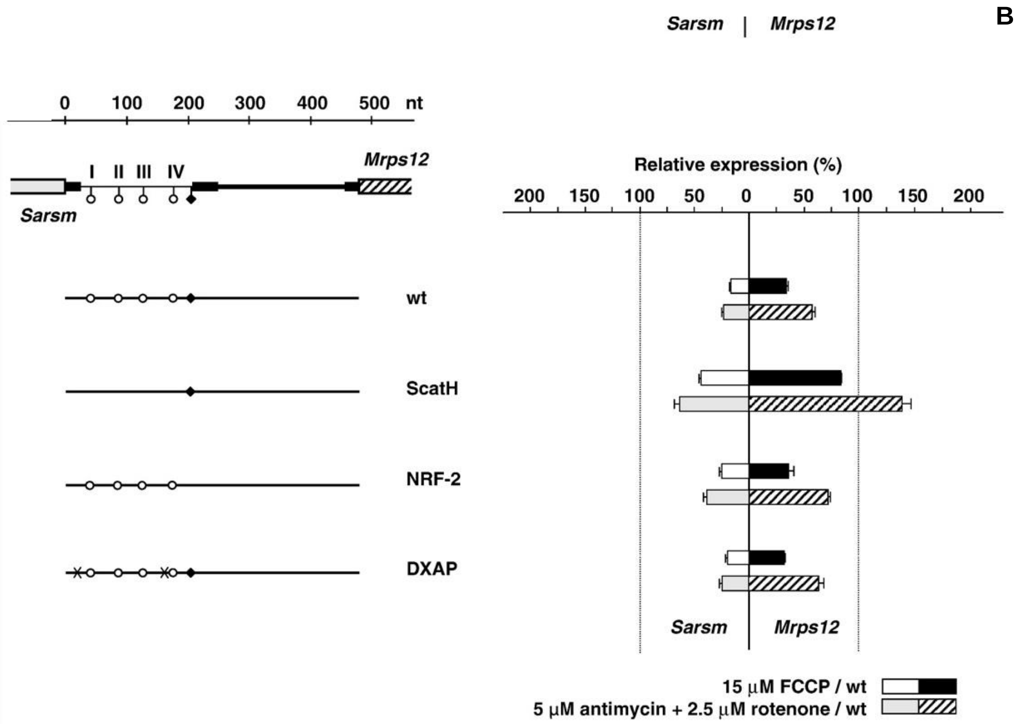


Figure 5.10. Effects of mutating the mouse *Mrps12/Sarsm* promoter on transcriptional response to mitochondrial OXPHOS stress. The various dual reporter constructs, shown diagrammatically at left using the same nomenclature as in Figure 5.3. with crosses denoting the sites of the additional mutations (I), were tested for promoter activity in 3T3 cells. Figure 5.10A: Cells pre-treated with FCCP (15 or 30 µM, which gave statistically indistinguishable results and are therefore pooled). The proportionate amount of induction of reporter activity by FCCP, typically 2–3-fold, varied, however, in individual experiments, which is assumed to be due to minor differences in the growth state of the cells. Data are therefore plotted for each mutant construct relative to the induction of the wild-type construct assayed in parallel in the same experiments. Plotted data (black bars in the *Mrps12* direction, white bars in the *Sarsm* direction) are means ± SD of the relative induction factor (%) for each construct, i.e. relative to the wild-type construct transfected and induced in parallel in at least six independent transfections in at least two replicate experiments. Figure 5.10.B: Cells pre-treated with 15 µM FCCP, or with 5 µM antimycin plus 2.5 µM rotenone, then reexposed to drug(s) 4 h after transfection. Plotted data (black or cross-hatched bars in the *Mrps12* direction, white or grey bars in the *Sarsm* direction) are means ± SD of the expression (%) of each construct, relative to that in nondrug-treated cells.

5.11 ROS production correlates with the induction of Mrps12/Sarsm promoter activity by mitochondrial stress (II)

To investigate possible components of the stress-mediated inducing signal, I treated NIH

3T3 cells with the same drugs that gave induction to varying degrees, then quantified mitochondrial ROS (superoxide) production using MitoSOX™ fluorescence (Figure 5.11). The potency of different drugs in producing the transcriptional response was well correlated with the amount of ROS production (FCCP > antimycin + rotenone > doxycycline = thiamphenicol). In HEK293 cells, the drugs produced similar effects on ROS production, even though the effects on transcription were opposite to those seen in NIH 3T3 cells.

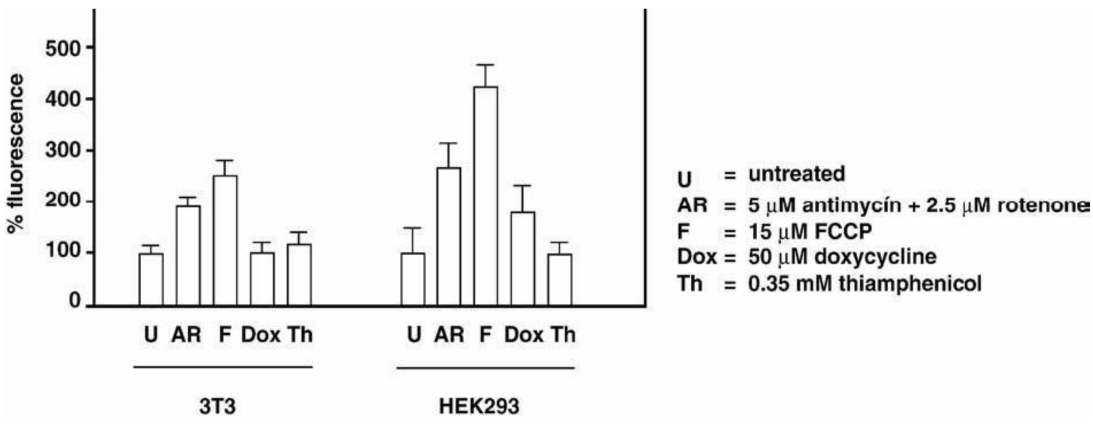


Figure 5.11. Effects of different drugs, as indicated, on MitoSOX™ fluorescence of NIH 3T3 and HEK293 cells. Cells were incubated in the drugs for the same times as used in pre-treatment for transcription assays. Means ± SD of three independent experiments.

6. Discussion

6.1 Sarsm/Mrps12 bidirectional promoter (I, II, III)

Bidirectional promoters are found in approximately 10% of the genes in mammalian genomes. However, only a small number have been properly characterized. I thus addressed my research to a detailed study of the *Mrps12/Sarsm* bidirectional promoter, in order to gain insight into how expression from this class of promoter is orchestrated. Since gene expression regulation, within a cell, can be highly affected by external stimuli, I studied the effects of toxins, interfering directly or indirectly with OXPHOS, on modulating *Mrps12/Sarsm* bidirectional promoter activity. For these purposes I used reporter analysis, EMSA, ChIP and *in vivo* footprinting, which showed a similar pattern of protein-binding to the promoter in cultured cells and solid tissues. I tested with reporter analysis the ability of NF-Y to recognize its core binding site in either orientation (i.e. as CCAAT or as ATTGG on the coding strand) and the role of NF-Y in regulating transcriptional directionality.

6.2 Transcriptional regulation of NF-Y (I, III)

I identified an array of four CCAAT box elements governing the extent and directionality of transcription of the oppositely transcribed genes *Mrps12* and *Sarsm* from their common intergenic promoter region in mice and humans. All four CCAAT boxes are oriented on the same strand, but those at each end appear to direct the transcriptional apparatus to initiate at the opposite end of the intergenic region. Computational analysis (performed by Andre Ribeiro and colleagues at Tampere University of Technology, III) showed that NF-Y type CCAAT boxes are found preferentially in bidirectional promoters in mouse and human genomes.

6.3 NF-Y in mouse (I)

CCAAT boxes I and IV of the mouse promoter show a good match to the consensus binding site for NF-Y (reviewed in Mantovani R, 1998) and I found that their destruction produces a strong effect on *Mrps12/Sarsm* promoter activity. In contrast, CCAAT boxes II and III of the array show a weaker affinity to the consensus binding site for NF-Y (Suppl. Figure 10 of I) and their destruction produced only minor changes in promoter activity, suggesting that they function most like accessory elements. However, promoter activity was mostly maintained leaving intact CCAAT boxes II and III both but destroying both CCAAT boxes I and IV. *In vivo* footprinting showed evidence of occupancy for all the four CCAAT boxes.

Both cultured cells and solid tissues in mouse presented a similar pattern of protein-binding occupancy, suggesting that differences in *Mrps12* and *Sarsm* mRNA expression level in different tissues must be brought about by post transcriptional control. NF-Y binding to the *Mrps12/Sarsm* promoter was confirmed *in vivo* by ChIP in NIH 3T3 cells. EMSA supershift using an antibody against the subunit NF-YA also gave evidence that NF-Y is the main binding factors capable of interacting with the promoter *in vitro*. Reporter analysis revealed different regulatory functions on promoter activity for each of the CCAAT boxes. However the four CCAAT boxes were inferred to be essential, since their simultaneous destruction abolished promoter activity. The destruction of one or more CCAAT boxes simultaneously, and in different combinations, confirmed a complex co-regulation pattern played by the four CCAAT boxes. The *Mrps12/Sarsm* promoter may represent a unique example of a bidirectional promoter regulated by the synergistic co-action of four CCAAT boxes.

NF-Y was shown previously to regulate housekeeping, cell type-specific and cell cycle-regulated genes (reviewed in Mantovani R, 1999). The presence of multiple CCAAT boxes within a promoter, whether unidirectional or bidirectional, could be to ensure that the genes are transcribed, since NF-Y can interact with other functionally important elements, and thus initiate and/or modulate transcription. NF-Y interacts with the TFIID complex, and thus with the TATA-binding elements, as well as with the steroid regulatory elements (SREBPs; reviewed in Mantovani R, 1999; Frontini M, et al. 2001). Multiple CCAAT boxes, in a bidirectional promoter, could function also to balance transcriptional directionality. I demonstrated in (III), by inverting or substituting one NF-Y binding site for another, that the overall promoter activity was changed but the directionality of transcription was maintained.

6.4 NF-Y in human (III)

Following the identification and functional characterization of the four CCAAT box array in mouse promoter, I addressed my studies to verify their possible functional similarities with the array of four CCAAT boxes localized also in the human promoter. Destruction of each single CCAAT boxes produced changes in the overall amount and directionality of transcription. Destruction of the two ‘end’ CCAAT boxes I and IV produced a pronounced change in promoter activity. CCAAT box II and III, whose destruction produced almost no change on promoter activity, appeared to have mainly an accessory function, similarly as seen in the mouse promoter.

Destruction of all four CCAAT boxes in the human *MRPS12/SARSM* promoter, did not completely abolish transcriptional activity, as previously seen in the mouse promoter, suggesting the possibility of a more complex regulation or the presence of other elements capable of sustaining the promoter activity. The regulation of the human promoter might be more complex, although it shares many common features with the mouse promoter, such as the similar overall organization and the finding by EMSA, that a probe lacking the four CCAAT boxes did not produce bands indicative of a clear DNA-protein binding, again identically to what was observed in mouse. However, dissimilarities among the human and mouse promoter regulation might not be linked to species differences, but instead could be due to cell-specific differences, similarly as I observed already analysing promoter response to mitochondrial stress.

Like the mouse promoter, the human *MRPS12/SARSM* promoter, NF-Y was shown to bind to the CCAAT boxes, *in vivo* by ChIP, and *in vitro* by EMSA supershift, using an antibody against NF-YA subunit. The inversion or substitution of one NF-Y- CCAAT box recognition sequence by another one of the array changed the overall promoter activity: however, transcriptional directionality was again maintained. This finding suggests that, as in mouse, NF-Y functions to regulate transcriptional directionality. In both species, the array of CCAAT boxes exhibited a complex pattern of co-regulation, with substantial promoter activity sustained leaving only one CCAAT box intact.

6.5 Possible roles of other regulatory factors (I)

In vivo footprinting detected occupancy also for the putative NRF-2 and AP-1 binding sites in mouse. Reporter analysis by destroying each of the binding sites had, however, only a modest effect on promoter activity. EMSA supershift using c-Jun antibody against the AP-1 factor did not show evidence of protein-DNA binding. EMSA did not show additional protein-DNA bands other than those attributed to NF-Y binding. The failure to confirm *in vitro* the *in vivo* evidence supporting an interaction of NRF-2 and AP-1 with the promoter, could be due to the transient interaction of those factors *in vivo* with NF-Y as a fundamental requisite for their binding to the promoter. NF-Y requires to interact with other factors to exert its transcriptional promoter activation or repression (Peng Y, et al. 2007). NF-Y can associate with the histone acetyltransferase PCAF and acquire activating functions, whereas NF-Y association to the histone deacetylase HDAC mediates its repressive function (Peng Y, et al. 2007).

Destruction of the recognition sequence for NRF-2 caused a decrease in *Mrps12/Sarsm* promoter activity of approximately 50%. However, NRF-2 could be still important for fine tuning of *Mrps12/Sarsm* transcription to physiological conditions, since it is known to be regulated by reactive oxygen species and to link mitochondrial function to the energy demand of the cell (Martin ME, et al 1996; Ammendola R, et al. 1995). In several experiments using EMSA, I found evidence of transient interaction between NF-Y and C/EBP, especially using liver nuclear extract, (Figure 5.5.B). The *Mrps12/Sarsm* promoter may thus be co-regulated by NF-Y and C/EBP as previously shown for mouse adiponectin (Park SK, et al. 2004), human microsomal epoxide hydrolase, EPHX1 (Zhu QS, et al. 2004) and mouse amelogenin (Xu Y, et al. 2006).

6.6 Cell-type differences in transcriptional responses to mitochondrial OXPHOS stress (II)

Gene expression is regulated at both the transcriptional and post transcriptional levels which act synergistically to fine-tune mRNA abundances. I tested the response of the *Mrps12/Sarsm* promoter to mitochondrial OXPHOS stress. *Mrps12* and *Sarsm* genes are key components involved in mitochondrial biogenesis and functionality, since their products are part of the mitochondrial transcriptional machinery. The *Mrps12/Sarsm* promoter clearly responded to mitochondrial OXPHOS stresses, which can activate or modulate cellular signals acting positively or overcoming basal proliferative signals, depending on the cell background. An increase in promoter activity, as seen in NIH 3T3 cells and to a lesser extent in Hela cells treated transiently with mitochondrial toxins, can be considered as a positive response to mitochondrial OXPHOS stress. I hypothesize that the cells responded positively by generating some signal to boost the expression of key genes which can maintain or restore mitochondrial function otherwise compromised by inefficient ATP production, respiratory electron flow or protein synthesis.

A different response to mitochondrial OXPHOS stress was obtained in HEK293, C2C12 and U2OS cells, where the *Mrps12/Sarsm* promoter was always downregulated, with only 10% of transcriptional activity remaining under higher drug doses, thus indicating toxicity or even a possible induction of apoptosis. Higher doses of mitochondrial inhibitors in HeLa cells or more prolonged drug exposure in NIH 3T3 cells could shift the response by repressing promoter activity, thus indicating that cells are finely poised and suggesting that perhaps all cells can respond in either way under appropriate stimuli.

The energy demand to cells is provided by oxidative phosphorylation and by glycolysis. Thus the response to OXPHOS stresses of a given cell may depend on the pathway predominantly used to produce ATP. In a cell which is highly dependent on OXPHOS for growth, mitochondrial stress may generate an anti-proliferative signal, which downregulates not only genes such as *Mrps12* and *Sarsm*, but the entire growth regulon, exposing the cells to the risk of apoptosis if externally induced signals continue to alter the cell's internal basal signals.

Cell-specific depletion of mtDNA under certain conditions could be a further element influencing the response to OXPHOS stresses. In HeLa cells mtDNA can be depleted by ethidium bromide treatment (Hayashi J, et al. 1991), and both 3T3 and C2C12 cells with mtDNA depletions have been generated by treatment with ditercalinium (Inoue K, et al.

1997). mtDNA can be depleted in HEK293 cells by expression of a dominant-negative variant of DNA polymerase gamma (Jazayeri M, et al. 2003). U2OS derived cells lacking mtDNA have not, to my knowledge, been reported yet.

Another response to mitochondrial OXPHOS stress could derive from a cell-specific ability to control an abnormal increase level of ROS, which are normally produced during basal metabolism taking part also in cell signaling. The transcriptional response of NIH 3T3 cells to different drugs was well correlated with their effects on mitochondrial ROS (superoxide) production. However, in HEK293 cells ROS level directly correlated with the suppression of *Mrps12/Sarsm* transcriptional activity.

Mitochondria dysfunction can have broad consequences for basal cellular signalling, such as mitochondrial control of calcium homeostasis or apoptosis (reviewed in Ryan MT & Hoogenraad NJ, 2007). However, during a mitochondrial OXPHOS stress condition, already characterized by an abnormal increase of ROS, some cells might find more convenient for their survival to increase the production of key elements of OXPHOS, which is the major source of ROS, thus restoring mitochondrial functions. Conversely other cells may respond to an increased level of ROS by further decreasing mitochondrial function, thus repressing the production of key components for OXPHOS, which are the source of ROS. However, when cells are overwhelmed by the damaging effects of excess ROS, activation of apoptotic pathways may overcome any other possible response.

6.7 Transcription factors involved in mediating responses to mitochondrial OXPHOS stress (II)

Stimulation of transcriptional activity following toxin treatments showed a signaling response more complex than expected, at least in mouse. I tested the possible involvement in stress response of other putative binding sites, such as NFAT, CREB, MYB and AP-1. However none of them showed evidence of involvement in the transcriptional stress response.

Reporter analysis using variant constructs did not identify a specific binding-site fully responsible for the promoter activity increase. Although the CCAAT box array is crucial for transcriptional activation, it was not found to be involved in promoter stimulation under FCCP treatment, as seen by the reporter analysis with all four CCAAT boxes destroyed and in contrast to previous evidence showing NF-Y mediation in transcriptional response to ER stress (Luo R, et al. 2008). The destruction of the four CCAAT boxes, however, did seem to attenuate promoter repression under prolonged drug treatment. This can result from a more accessible interaction between the promoter and other factors which might be able to sustain part of the transcription. The stronger down regulation might be mediated by the CCAAT box binding elements, as an anti-proliferative mechanism to ensure that the available energy is used mainly for repairing damage than for cell division, thus exhibiting features related to replicative senescence which is characterized by an overall loss of dividing potential (reviewed in Matuoka K & Chen KY, 2002).

In addition, other factors which can interact with the CCAAT box could be involved in the promoter response to mitochondrial stress. The transcription factor C/EBP family has been shown to be involved in the ER stress response (Zinszner H, et al. 1998). Destruction of the NRF-2 binding site slightly attenuated *Mrps12/Sarsm* downregulation under prolonged drug treatment. However, the response attenuation associated with destruction of the NRF-2 and CCAAT boxes could be explained on the base that toxin treatments are destroying or inactivating other factors interacting with them and essential for sustaining high-level transcription from the promoter.

7. Conclusions:

The goal of my PhD research study was to characterize the bidirectional promoter for the human and mouse homologous genes mitoribosomal protein S12 (*Mrps12*) and mitochondrial seryl-tRNA synthetase (*Sarsm* or *Sars2*). *Mrps12* and *Sarsm* are nuclear genes that encode components of the mitochondrial translational apparatus. In humans, a number of mutations are reported to affect the mitochondrial translational apparatus and to be responsible for severe metabolic diseases. I commenced by mapping the mouse and human *Mrps12/Sarsm* intergenic region, including also the *Mrps12* UTR sequence, in order to localize the main regulatory regions. Some specific binding sites and their binding factors which mainly regulate the promoter activity were found by combining several methods. Promoter functionality was studied using a dual luciferase reporter vector. Protein factor-DNA binding was studied using EMSA, in-*vivo* footprinting and Chip. The project continued then by analyzing how the promoter activity responds to an induced mitochondrial stress, created by treating several cell-type cultures with toxins which differently affect mitochondrial functions. A biphasic promoter activity modulation, which was up or down regulated depending on cell-type and/or time treatment with toxins, was followed by testing the possible involvement of some *cis*-acting elements on the transcriptional activity response over an induced mitochondrial stress.

Mrps12/Sarsm promoter appeared to be regulated by an array of four CCAAT boxes interacting with the transcription factor NF-Y in both human and mouse.

In mammals, the transcriptional regulation of *Mrps12* and *Sarsm* by NF-Y represents a rare example in which the expression of genes encoding key component proteins of mitochondrial translational machinery, thus involved also in the organellar biogenesis and function, is regulated by this factor.

NRF-2 and NFR-1 were previously shown to have a key role in the regulation of nuclear genes encoding components of the mitochondrial OXPHOS system in mammals. However, the putative NRF-2 in *Mrps12/Sarsm* promoter seemed to have mainly an accessory function and probably it is involved in fine-tuning of *Mrps12/Sarsm* transcription.

Mitochondrial OXPHOS stress, induced by toxins inhibiting specifically the organellar function, produced multiple effects on promoter activity in the different mouse and human cell lines I tested and at different times of exposure. Promoter activity could be stimulated or suppressed.

The inversion or substitution of one NF-Y- CCAAT box recognition sequence by another one of the array changed the overall *Mrps12/Sarsm* promoter activity: however, transcriptional directionality was again maintained. This finding suggests that, both in mouse and human, NF-Y functions to regulate transcriptional directionality.

An appropriate knowledge of the component pathways and their co-interaction in nucleus-mitochondria signalling could have application in therapy, including the control of the apoptotic pathway in cancer, and the possibility to delay ageing related loss of mitochondrial function.

Deficiency at the mitochondrial translational apparatus represents, so far, a rare human disease, but at present an increasing number of cases is reported. The molecular mechanisms of this class of pathology are partly understood, but the reasons why the defect is manifested in such a tissue-specific manner remain unknown. Therefore, an animal model, manifesting a deficiency at the mitochondrial translational apparatus, would represent a useful tool also for attempting a therapeutic strategy.

8. Additional Work

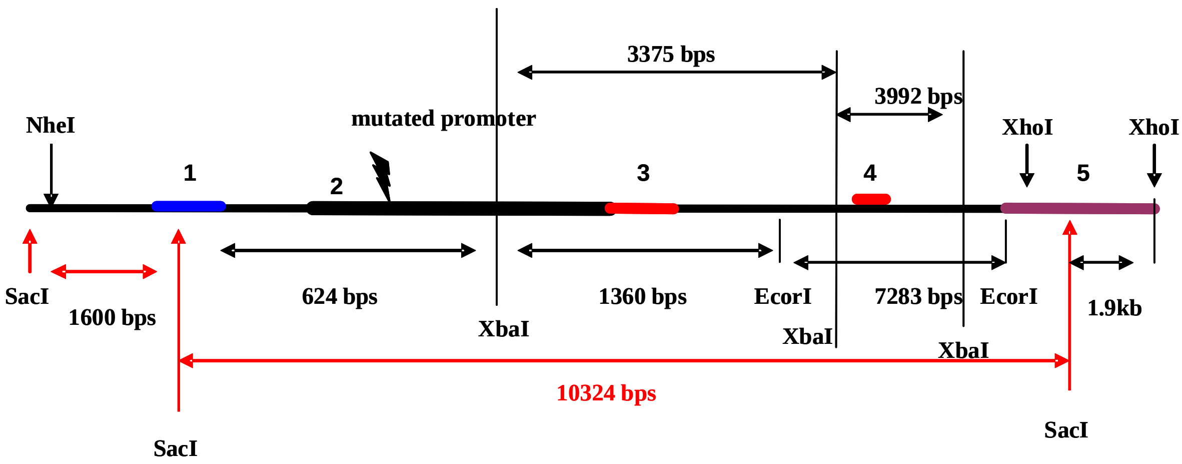
In addition to the published work, I was also involved in the characterization of so-called *tko' Mrps12*^{L85H}mice, mice with a single substitution (L85H) at a conserved residue of the protein. The *tko* mice were created before my arrival in the lab by Dr. Zahid H. Shah. The aim of the project was to attempt to create a mouse model with a mitochondrial translational pathology, similar to that obtained in this lab using *tko* mutant flies which carry a homologous mutation in the fly *tko* gene, encoding *Mrps12*. Mutant *tko* flies exhibit developmental delay, defective response to sound and are hypersensitive to doxycyclin. However, *tko* mice did not exhibit evident pathological phenotype even during ageing. Oxygen consumption measurement using isolated mitochondria from brain, liver, kidney and heart did not differ between mutant and wild-type mice. Similarly no differences were found in the biochemical measurement of the electron transport chain complex activities. My main contribution was to optimize the biochemical measurement of the oxygen consumption using mitochondria from many mouse tissues and the measurement of electron transport chain enzymatic activity: complex I-II-III-IV and citrate synthase.

So called *Humppa* mice, accidentally created during the ES gene targeting work, were found to have a large deletion in chromosome 1. *Humppa* mice exhibited a curious phenotype: cycling movement, balance disorders, growth retardation and a severe reduction of oxygen consumption from mitochondria of the liver.

Finally, I attempted to create a mouse model deficient at the mitochondrial translation apparatus. Once the binding sites playing an important role in the regulation of *Mrps12* and *Sarsm* gene expressions were found, I used this information to try to manipulate the expression of the two genes by creating knock-in mice. Based on the functional promoter analysis obtained from the luciferase assays, I made targeting constructs for three different knock-in mice by destroying one or more CCAAT boxes in each of the constructs. In one construct I down regulated the expression of both genes (*Sarsm/Mrps12*). In the second construct I up regulated the promoter activity on the direction of *Sarsm*, but *Mrps12* expression was unaffected. In a third construct I slightly reduced the promoter activity in both directions.

I created the targeting constructs, carrying the desired mutated promoter, together with the classical positive (hygromycin-resistance) and negative (HSV-TK) selectable markers for homologous integration. The constructs were electroporated into *ES* cells (work done in transgenic mice core facility). For the southern blot screening the *ES* genomic DNA was digested with restriction enzymes. I used internal and external probes from the targeting construct to verify for successful homologous recombination. However, none of the screened *ES* colonies presented a correct homologous recombination of the vector sequence. A specific gene targeting would be improved by 'ending' the DNA of the contructs with exons.

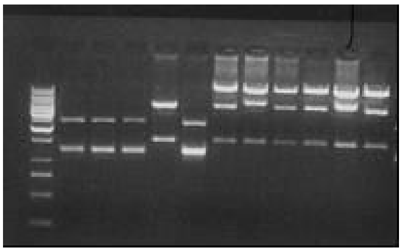
Knock-in Construct strategy to create transgenic mice, description of the main restriction sites



1: sarsm exon 1
2: promoter region
3 and 4: mrps12 exon 1 and 2
5: Thymidine kinase

Digesting the contruct with *SacI* produced 3 fragments as seen in run SI. The bands are 1.6 kb, 10,3 kb and 4.9 kb (which includes also the vector sequence)

SI



Digestion of the construct by *SacI*: DNA Ladder Mix is SM0331/2/3 from *FERMENTAS*,

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11. ORIGINAL PUBLICATIONS