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Glucocorticoids induce mitochondrial gene transcription in HepG2 cells Role of the mitochondrial glucocorticoid receptor

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ABSTRACT

Glucocorticoids are major regulators of a plethora of cellular functions, acting on target cells through glucocorticoid receptors (GR) and modulation of gene transcription, among other mechanisms. One main site of action of glucocorticoids is the hepatocyte, which responds to the hormonal stimulus with induction of several proteins among them enzymes of oxidative phosphorylation (OXPHOS), both nuclearly and mitochondrially encoded. The induction of OXPHOS is regarded as a result of a nuclear action of the receptor on the respective nuclear genes and on genes encoding mitochondrial transcription factors. The presence of GR in mitochondria and of sequences in the mitochondrial genome similar to glucocorticoid responsive elements, suggested a direct action of GR on mitochondrial transcription. We demonstrate in HepG2 hepatocarcinoma cells specific binding of GR to the regulatory D-loop region of the mitochondrial genome and show that dexamethasone induces the mitochondrial transcription factors A, B1, and B2, the mitochondrial ribosomal RNA, and several mitochondrially encoded OXPHOS genes. Applying α-amanitin, the specific inhibitor of DNA-dependent RNA polymerase II, the dexamethasone-induced transcription of the mitochondrial genes can still proceeds, whereas the DEX effect on transcription of the mitochondrial transcription factors is suppressed. Moreover, HepG2 cells overexpressing mitochondrial targeted GR showed increased RNA synthesis, cytrochrome oxidase subunit I protein expression, and mitochondrial ATP production. We conclude that glucocorticoids can stimulate directly mitochondrial transcription by the mitochondrially localized GR, affecting OXPHOS enzyme biosynthesis. This takes place in addition to their action on mitochondrial genes by way of induction of the nuclearly encoded mitochondrial transcription factors.

1. Introduction

Glucocorticoids are important regulators of basic cellular processes, such as metabolism, growth, differentiation, and survival [1]. Their mode of action in target cells has received considerable interest and it is now clear that they can act in various ways, e.g., on the gene level by way of cognate receptors and on cytoplasmic processes by way of membrane receptors. One of the first model systems which led to the demonstration that glucocorticoids act through cognate cytoplasmic receptors to stimulate transcription of genes and induce the respective proteins was that of cortisol inducing in the rodent liver enzymes involved in gluconeogenesis [2–6].

In these and later studies, a bulk increase of HnRNA containing the pre-mRNAs of the induced genes, ribosomal RNA and t-RNA was observed [3–5], which could not account for the increased mRNA synthesis corresponding to the transcription of the few genes, identified as glucocorticoids-induced genes. Recent results from studies involving bio-array analyses [7–9] have shown that the glucocorticoid effect on the hepatocyte and on other target cells encompasses several genes, and, in part, justifies the observed overwhelming increase in nuclear RNA synthesis. Mitochondrial RNA synthesis is also stimulated by glucocorticoids [3,6]. The hormonal induction process is highly energy consuming, and the cell makes efforts to guarantee the replenishment of its energy reserves. Therefore, it is understandable that the glucocorticoid induced mitochondrial RNA synthesis can be correlated to this energy regeneration process, as the mitochondrial genome encodes subunits of OXPHOS enzymes and RNAs participating in the protein synthesis in mitochondria. Due to the fact that some OXPHOS are encoded in the nuclear genome and others in the mitochondrial genome it is assumed, in analogy to the action of thyroid hormones on liver OXPHOS [reviewed in 10] that the hormonal stimulus affects the transcription of the mitochondrial encoded OXPHOS indirectly, by inducing nuclear signals, such as mitochondrial transcription factors,

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which then, secondarily, positively regulate transcription of the mitochondrial genome. However, the demonstration of the mitochondrially localized glucocorticoid receptor and the presence of sequences with strong homology and characteristics of glucocorticoid response elements (GRE) in the mitochondrial genome [11], raised the possibility of a direct effect of the mitochondrial GR on mitochondrial transcription, in addition to the indirect nuclear pathway [12,13].

We now present data demonstrating that dexamethasone (DEX), a synthetic glucocorticoid, induces in HepG2 cells the mitochondrial transcription factors A, B1, and B2 (TFAM, TFB1M, TFB2M), the 12 S and 16 S mitochondrial ribosomal RNA and several mitochondrial DNA (mtDNA) encoded genes. Applying α-amanitin, the inhibitor of the DNA-dependent RNA polymerase II, we show that the inducing effect of dexamethasone on the mitochondrial ribosomal RNA and the mtDNA encoded genes can still take place in the presence of the inhibitor, although transcription of the nuclearly encoded mitochondrial transcription factor genes is reduced. Furthermore, we demonstrate by chromatin immunoprecipitation (ChIP) analysis, binding of GR to sequences with strong homology to GREs in the D-loop regulatory region of the mitochondrial genome and provide evidence for an involvement of the increased mitochondrial GR expression in promoting mitochondrial RNA synthesis and mitochondrial ATP production.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL. Molecular weight protein markers and complete protease inhibitors cocktail were purchased from Fermentas and Roche, respectively. Lipofectamin 2000 and MitoTracker Red CMXRos (CMX) were from Invitrogen. Geneticin (G418) was from Calbiochem. All other chemicals including α-amanitin were purchased from Sigma-Aldrich.

2.2. Cells culture — HepG2 cells stably expressing a mitochondrial targeted GFP-GR fused protein (HepG2-mtGFPGR cells)

Human HepG2 cells were maintained in DMEM, supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

For stable cell lines generation, at day 1 before transfection, HepG2 cells were plated in 60-mm culture dishes so that they reached 50–70% confluency at the time of transfection. To obtain stable expression of the human mitochondrial GR the following sequence: atggctcagcgacttcttctgaggaggttcctggcctctgtcatctccaggaagccctctcagggtcagtggccacccctcacttccagagccctgcagaccccacaatgcagtcctggtggcctgactgtaacacccaacccagcccggacaatatacaccacgaggatctccttgaca, which encodes a mitochondrial targeting peptide was inserted in frame with the enhanced green fluorescence protein (EGFP) gene between NheI and AgeI sites of the $pEGFPC_2$ vector (Clontech) to produce the $pmtEGFPC_2$ construct. Subsequently, the human glucocorticoid receptor gene (X03225 accession no.) was inserted into the $pmtEGFPC_2$ construct at the BamHI site ($pmtEGFPC_2$-GR). The produced construct was transfected into the HepG2 cells using Lipofectamine 2000, according to the manufacturer's instructions. Control cells (HepG2-mtGFP cells) were prepared by transfection of the pmtEGFPC2 construct. After 24 h incubation at 37 °C, cells were thrypsinized and passaged at a 1:5 dilution in selective growth medium [DMEM supplemented with 10% (v/v) FBS, containing 1.5 mg/ml Geneticin (G418)]. On the next day, the medium in all plates was replaced with fresh selective growth medium and cells were cultured for 2 weeks. G418 resistant colonies were expanded and cloned independently and analyzed by immunofluoresence, Western blot analysis, and immunoprecipitation to confirm the expression of the mitochondrial GFP (mtGFP) and GFP-GR (mtGFPGR) proteins.

2.3. Inhibition of nuclear RNA synthesis — α-amanitin treatment

HepG2 cells grown on 6 well plates were treated with 10 μg/ml α-amanitin, for 5 h, at 37 °C. Control as well as α-amanitin treated cells were stimulated either with 10^{-6} M DEX (diluted in Ethanol), or equal volume of ethanol for 1 h at 37 °C. Subsequently, cells were washed with phosphate buffer saline and total RNA was extracted using Trizol followed by DNase treatment (Promega) and reverse transcription into cDNA, using random primers and superscript II reverse transcriptase (Invitrogen). Expressed levels of mRNA were quantitated using real-time RCR which was performed after mixing the cDNA with SYBR GreenER qPCR super mix Universal (Invitrogen) and appropriate primers. Products were quantitated with a Chromo4 Real-Time System (Bio-Rad). Conditions for PCR were: 52 °C for 2 min, 95 °C for 2 min, 35 cycles of 95 °C for 15 s and 60 °C for 40 s, followed by 60 °C for 10 min. Primers for mitochondrial encoded OXPHOS genes, cytochrome oxidase subunit IV (COX IV), and 12 S and 16 S RNA are shown in Table 1. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a nuclear reference gene are: GAPDH forward: catgagaagtatgacaacagcct; GAPDH reverse: agtcctttccacgataccaaagt [13].

2.4. ChIP assay

HepG2 cells, grown in 15 cm Petri dishes were first cross-linked with formaldehyde (final concentration, 1%) for 10 min at room temperature, and then the reaction was stopped with glycine (0.125 M) for 5 min at room temperature. Cells were then rinsed twice with ice-cold PBS, collected into ice cold PBS supplemented with a protease inhibitor cocktail (Roche). After centrifugation the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA 50 mM Tris–HCl pH 8.1 and protease inhibitors). The lysates were sonicated on ice at 30% of maximum of power (Sonics' Vibra-cell), to yield DNA fragments of 500 to 200 bp in length. After preclearance of the resulting chromatin with salmon sperm DNA-protein A/G agarose 50% slurry (Upstate Biotechnology) for 1 h at 4 °C with agitation, immunoprecipitation was performed overnight at 4 °C either with a GR specific monoclonal antibody (2F8) produced and kindly provided by Dr. M. Alexis (Hellenic Research Foundation) or normal mouse IgG as control. Agarose beads were pelleted by centrifugation and washed extensively according to Upstate Biotechnology instructions. Finally the bound DNA fragments were eluted in freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$), purified, concentrated, amplified and quantified either by semiquantative PCR or by real-time PCR. Binding to the putative mitochondrial hormone response elements in the D-loop, 12 S, NADH-CoQ-reductase subunit I (ND I) region was checked

Table 1
Primers for human mitochondrial OXPHOS enzymes, COX IV, 12 S, and 16 S mitochondrial RNA.

	Forward	Reverse
ND I	atggccaacctcctactcctcatt	ttatggcgtcagcgaagggttgta
ND II	ccatctttgcaggcacactcatca	attatggatgcggttgcttgcgtg
ND III	gccctacaaacaactaacctgcca	ataggccagacttagggctaggat
ND IV	agctccatctgcctacgacaaaca	taagcccgtgggcgattatgagaa
ND V	cacagcagccattcaagcaatcct	acctaattgggctgatttgcctgc
ND VI	ataggatcctcccgaatcaaccct	aggattggtgctgtgggtgaaaga
COX I	accctagaccaaacctacgccaaa	taggccgagaaagtgttgtgggaa
COX II	acagatgcaattcccggacgtcta	ggcatgaaactgtggtttgctcca
COX III	tcacttccactccataacgctcct	gtgttacatcgcgccatcattggt
ATP6	acattactgcaggccacctactca	acgtaggcttggattaaggcgaca
ATP8	accgtatggcccaccataattacc	tttatgggctttggtgagggaggt
Cyt b	agtcccaccctcacacgattcttt	agtaagccgagggcgtctttgatt
12 S	aaactgctcgccagaacactacga	tgagcaagaggtggtgaggttgat
16 S	taccctcactgtcaacccaacaca	ttaaacatgtgtcactgggcaggc
COX IV	agaaagtcgagttgtatcgcatt	gataacgagcgcggtgaaac

[11]. The primer sequences and the positions from transcription start sites are as follows: D-Loop Forward (−16,549 bp): TCGCTCCGGGCCC ATAACACTT; D-Loop Reverse (−16,449 bp): GGAACGTGTGGGCTATT TAGGCTT; 12 S Forward (−1253 bp):AAAGGACCTGGCGGTGCTTCA TA; 12 S Reverse (−1166 bp):GAGCAAGAGGTGGTGAGGTTGAT; ND I Forward (−4168 bp):AGGAACAACATATGACGCACTCTCCC; ND I Reverse (−4036 bp):GTGTATGAGTTGGTCGTAGCGGAA. Recovery of DNA was calculated as percentage of input material of the immunoprecipitation. Myoglobin gene was used as a negative control for GR chromatin binding. The primers sequences for myoglobin are; Forward:TGGCAC CATGCTTCTTTAAGTC; Reverse:AAGTTTGACAAGTTCAAGCACCTG).

2.5. In organello RNA synthesis

Mitochondrial RNA synthesis was measured in faithfully transcribing isolated mitochondria from HepG2 cells as previously described [14–16]. Briefly, freshly isolated crude mitochondria (10,000×*g* pellet) (produced as previously described [17]) were incubated at a final mitochondrial protein concentration of 2 mg/ml in 0.5 ml of incubation buffer containing 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris–HCl (pH 7.4) and 1 mg of bovine serum albumin. 20 μCi of [α-^{32}P]UTP was added to the medium, and incubation was maintained at 37 °C for 60–90 min. For various time intervals mitochondrial samples were pelleted at 13,000×*g* for 2 min and the supernatant with the non incorporated [α-^{32}P]UTP was removed. Mitochondria were washed once and then lysed in 20 mM Tris pH: 7.5 supplemented with 2% SDS. The acid-insoluble material was precipitated on a glass fiber filter with 5% trichloroacetic acid. Filter were washed five times with 5% TCA, twice with ether, and dried. Radioactivity was measured by liquid scintillation counting. The [α-^{32}P] UTP was linear up to 60–90 min.

2.6. Mitochondrial ATP content measurements

Mitochondria from HepG2-mtGFP and HepG2-mtGFPGR cells were freshly isolated as previously described [17]. ATP from isolated mitochondria was extracted with 1% TCA and ATP content was measured in a luminometer (Berhold) by bioluminescence using the luciferin-luciferase reaction-Kit (Enliten, Promega) following the manufacturer's instructions.

2.7. Immunoprecipitation-Western blot analysis

Mitochondria from HepG2-mtGFPGR cells were lysed in RIPA buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) supplemented with protease inhibitors and sonicated for 3×20 s. Insoluble material was removed by centrifugation at 13,000×*g* for 15 min and the supernatants were preincubated for 1 h, with 20 μl protein A sepharose, at 4 °C. Samples were centrifuged for 2 min at 1500×*g*, and the supernatants were further incubated with the primary antibodies mouse anti-GR (2F8) or mouse anti-GFP (Roche). Samples incubated with normal mouse IgG were used as control. 20 μl of protein A sepharose was added to each sample and incubated for 3 h, at 4 °C. Protein A sepharose was pelleted by centrifugation at 1500×*g* for 2 min and washed three times in PBS. The samples were boiled in SDS-PAGE loading buffer, in the presence of 5% β-mercaptoethanol, for 4 min and centrifuged at 1500×*g*. The resulting supernatants were loaded on a 10% SDS-PAGE gel, electrophoresed and Western blotted with specific rabbit polyclonal antibodies against GR (GR-H300) commercially provided by Santa Cruz Biotechnology. Cytochrome oxidase subunit I (COX I) protein synthesis was evaluated by Western blot analysis. Equal amount of total protein extracts from HepG2-mtGFP and HepG2-mtGFPGR cells were electrophoresed on a 10% SDS-PAGE gel and Western blotted with specific mouse monoclonal antibodies against COX I (Invitrogen). Protein expression levels were normalized to β-actin protein expression using specific monoclonal antibodies commercially provided by Sigma.

2.8. Immunofluorescence-confocal microscopy

Cells grown on coverslips were incubated for 30 min at 37 °C with 200 nM CMX in the presence or absence of 10^{-6} M DEX; washed 3×5 min with PBS, fixed for 10 min in ice-cold methanol and transferred to ice-cold acetone for 2 min. For nuclear staining specimens were incubated with 10 ng/ml Hoechst 33342 in PBS, for 30 min at room temperature. Antibodies against GR (GR-300) were used for GR detection. Secondary Alexa 488-conjugated antibodies were also used and purchased from Invitrogen. The specimens were washed in PBS and mounted in anti-fading medium [18]. Images were taken at 23–24 °C using an inverted laser-scanning confocal microscope (TCS SP5; Leica) equipped with a 63× NA 1.3 oil immersion objective lens (Leica) and LAS-AF acquisition software (Leica).

2.9. Statistical analysis

All results are expressed as mean ± SD (n = 3–5). Data were analyzed both by independent *t*-test and Mann–Whitney test. Differences were considered significant at a two tailed P value <0.05.

3. Results

3.1. Production of HepG2 cells stably overexpressing mitochondrial targeted GFP-GR

In order to elucidate the role of the mitochondrial GR in mitochondrial functions and particularly in the mitochondrial transcription, HepG2 cells stably expressing the human GR fused with a mitochondrial targeted green fluorescence protein at its N terminus were generated as described in material and methods. Cells stably expressing mitochondrial GFP were also produced and used as control.

The presence of endogenous mitochondrial GR in HepG2 cells, in the presence or absence of DEX, is demonstrated in Fig. 1A and B corroborating previous findings [17,19]. Cells stably overexpressing GR fused with the mitochondrial targeted GFP protein (HepG2-mtGFPGR) are shown in Fig. 1C, D, and E. Mitochondrial targeted GFPGR (Fig. 1 C1, D1, E1) exhibited the same pattern of staining and colocalized with the mitochondrial marker CMX (Fig. 1 C2, D2, E2) verifying the mitochondrial localization of the expressed protein in the HepG2-mtGFPGR cells (Fig. 1 C4, D4, E4). As was expected, the presence of dexamethasone induced nuclear translocation of the endogenous GR (Fig. 1 B1). Dexamethasone did not cause any changes in the distribution of the stably expressed mtGFPGR protein (Fig. 1 D1). HepG2 cells stably expressing mtGFPGR showed a 2–3 fold increase in the mtGFPGR expression compared to endogenous mtGR as indicated by Western blot analysis (Fig. 1F, lane 3). Western blot analysis of GR in subcellular fractions of HepG2-mtGFPGR cells as well as immunoprecipitation analysis of GR in mitochondrial fractions using specific antibodies against a) GR (Fig. 1F, lane 6) or b) GFP proteins (Fig. 1F, lane 7) verified the mitochondrial localization of the mtGFPGR protein (Fig. 1F lanes 3, 6, 7) and its absence from the cytosolic fraction (Fig. 1F lane 2). Interestingly, the co-immunoprecipitation of the endogenous, approximately 90 kDa, mitochondrial GR protein with the mtGFPGR protein by the anti-GFP antibodies, was also observed. HepG2 cells stably overexpressing mitochondrial targeted GFP protein (HepG2-mtGFP cells) were also produced, checked for the mitochondrial localization of the mtGFP protein (data not shown), and used as control.

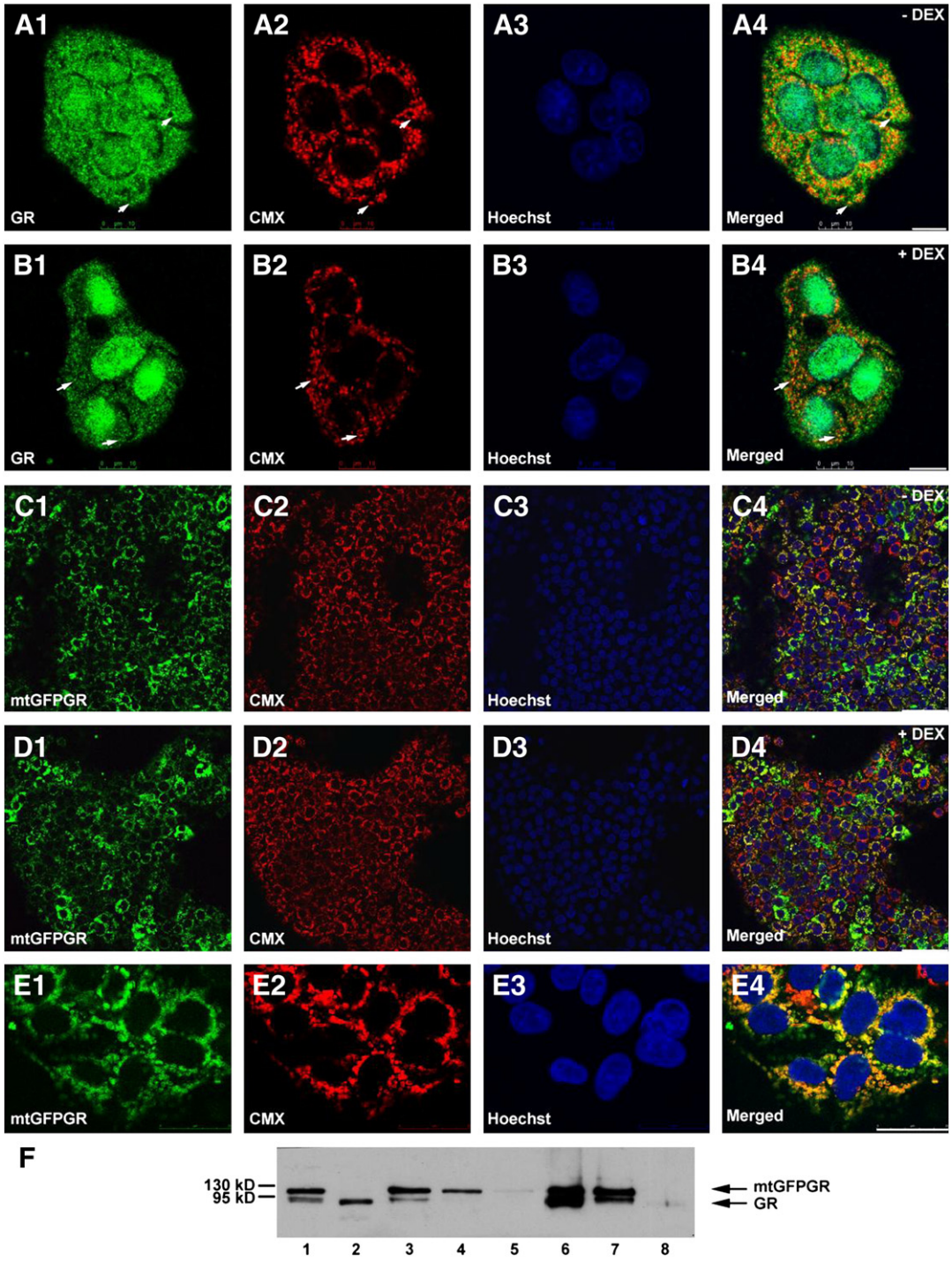

Fig. 1. Mitochondrial localization of glucocorticoid receptor in HepG2 cells. Mitochondrial localization of GR in HepG2 cells in the absence (A) or presence (B) of DEX. Methanol-aceton fixed specimens pretreated with CMX (A2, B2) were subjected to indirect immunofluorescence staining of GR using the GR-H300 polyclonal antibodies (A1, B1). White arrows indicate colocalization of GR with CMX. Expression and mitochondrial localization of mtGFPGR in methanol-aceton fixed specimens of HepG2-mtGFPGR cells in the absence (C, E) or presence (D) of DEX. C1, D1, and E1: mtGFPGR localization; C2, D2, and E2: CMX mitochondrial staining; A3, B3, C3, D3, and E3: nuclear staining of HepG2 cells with the Hoechst 33342 dye; and A4, B4, C4, D4, and E4: merged images. Bars in A–B, C–D and E indicate 10, 50 and 25 μm, respectively. F: Western blot analysis of GR in subcellular fractions of HepG2-mtGFPGR cells and immunoprecipitation analysis of mtGR and mtGFPGR in mitochondrial extracts from HepG2-mtGFPGR cells. Detection of GR in HepG2-mtGFPGR cells was achieved by Western blot analysis, applying the GR-H300 antibodies. The anti-GR (2F8) and anti-GFP antibodies were applied in immunoprecipitation studies; lane 1: total cell extract; lane 2: post mitochondrial supernatant; lane 3: 10% of mitochondrial extracts used for immunoprecipitation; lanes 4 and lane 5: 10% of unbound mitochondrial proteins immunoprecipitated with the anti-GR and anti-GFP antibodies, respectively; and lanes 6, 7, and 8: immunoprecipitated mitochondrial proteins with the anti-GR, anti-GFP antibodies, and normal mouse IgG, respectively.

3.2. Effect of DEX on RNA synthesis of nuclear encoded mitochondrial transcription factors and of mitochondrial OXPHOS genes in HepG2 cells

HepG2 cells were induced with 1×10^{-6} M dexamethasone and the transcription of the genes for the mitochondrial transcription factors TFAM, TFB1M, TFB2M, the nuclear and mitochondrial OXPHOS genes, and the mitochondrial 12 S and 16 S ribosomal RNA was assayed by RT-PCR (see Materials and methods). The results are shown in Fig. 2. In HepG2 cells the presence of dexamethasone led to increased transcription of several genes, among them the mitochondrial transcription

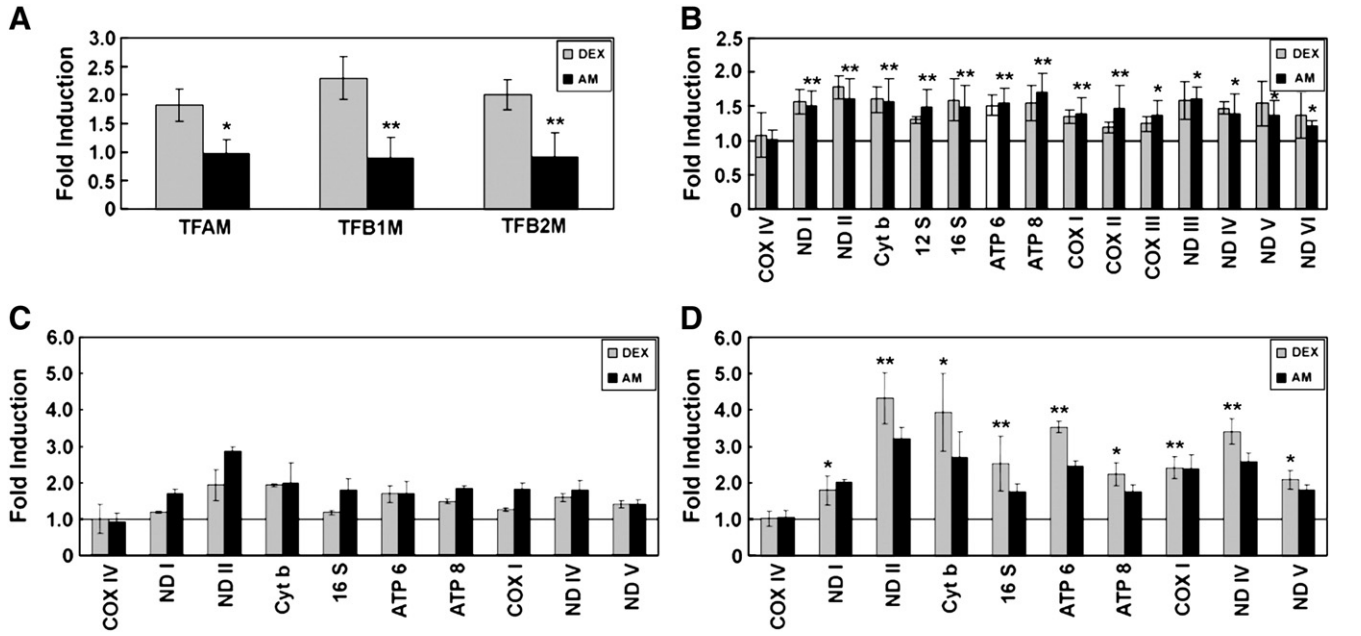

Fig. 2. Effect of DEX on mitochondrial RNA transcription. A, B) HepG2 cells grown on 6 well plates were treated with 10 μg/ml α-amanitin, for 5 h at 37 °C. α-amanitin treated as well as non treated cells were further incubated in the presence or absence of 10^{-6} M DEX diluted in ethanol, for 1 h at 37 °C. Control cells were incubated with equal volume of ethanol. Subsequently cells were washed with phosphate buffer saline and subjected to RNA extraction. Expressed mRNA levels of A) nuclear encoded mitochondrial transcription factors and B) COX IV, mitochondrial encoded OXPHOS and mitochondrial ribosomal RNAs were quantified by real-time PCR. HepG2-mtGFP (C) and HepG2-mtGFPGR cells (D) were also subjected to α-amanitin treatment as described above, and subsequently stimulated with DEX or equal volumes of ethanol, for 1 h at 37 °C. mRNA levels measurements were performed applying real-time PCR. Expressed levels of GAPDH were measured for the normalization of the results. Results are expressed as fold induction of the indicated DEX induced gene expression, in the absence (DEX) or presence of α-amanitin (AM), compared to the GAPDH gene expression, and represent the mean ± SD of at least three individual experiments. $^{*}P<0.05$; $^{**}P<0.005$; A. Compared to DEX induction in the absence of α-amanitin; B. Compared to α-amanitin effect on nuclear encoded COX IV gene; D. Compared to DEX induction in HepG2-mtGFP cells.

factors examined (Fig. 2A), the mitochondrial 12 S and 16 S RNA and several mitochondrial OXPHOS genes such as NADH-CoQ-reductase subunits I–IV (ND I–IV), cytochrome b (Cyt b), ATP-synthase 6 and 8 (ATP 6 and ATP 8), and COX I (Fig. 2B).

In order to evaluate the contribution of the dexamethasone induced increase in transcription of the mitochondrial transcription factors and the mitochondrial encoded genes, we repeated the experiments depicted in Fig. 2 in the presence of concentrations of α-amanitin known to inhibit HnRNA synthesis and also inhibit dexamethasone induction of gluconeogenic liver enzymes [20,21]. α-amanitin is an inhibitor of DNA dependent RNA polymerase II. Administration of amanitin to animals or cells leads to inhibition also of other RNA types, such as ribosomal RNA, however mitochondrial RNA synthesis is not affected [20,22,23]. As demonstrated in Fig. 2A, α-amanitin almost completely blocked the dexamethasone effect on TFAM, TFB1M and TFB2M transcription, (exhibiting induction of transcription of those genes similar to the GAPDH gene used for the normalization of the results). In contrast, in the presence of α-amanitin the dexamethasone induced expression of the mitochondrial encoded genes could still proceed (Fig. 2B). In addition, the effects of dexamethasone on OXPHOS gene expression, in HepG2-mtGFPGR cells, were more pronounced, particularly genes encoding ND II, Cyt b, 16 S, ATP 6, ATP 8, COX I, ND IV ND V showed a 3–4 fold induction and which was 1.5–2 fold of the induction observed in HepG2-mtGFP cells (Fig. 2C, D). α-amanitin occasionally reduced transcription of the mitochondrial genes, but still permitted dexamethasone induction of these genes to an extent of 70–100% in respect to cells not treated with the α-amanitin.

3.3. Effect of mitochondrial GR on mitochondrial transcription in an organello system

The DEX induced mitochondrial RNA expression which was more pronounced in HepG2-mtGFPGR cells prompted us to examine the mitochondrial RNA synthesis in a faithfully transcribing mitochondrial organello system. Isolated mitochondria from HepG2-mtGFPGR cells showed increased capacity to synthesize RNA compared to equal amounts of mitochondria from control HepG2-mtGFP cells (Fig. 3A). The presence of dexamethasone causes an approximately 1.5–2 fold induction in mitochondrial transcription of isolated mitochondria of HepG2-mtGFPGR cells (Fig. 3B) whereas, in the presence of the GR antagonist, RU486, the effect of DEX on the mitochondrial transcription is diminished.

3.4. Binding of GR to the mitochondrial DNA

The effects of dexamethasone on mitochondrial gene transcription in an organello system and in HepG2 cells, even in the presence of a-amanitin, supported the direct effect of GR on mitochondrial transcription, presumably as a consequence of binding of the receptor to the mitochondrial genome. To test this assumption a ChIP analysis of GR binding to mitochondrial DNA, particularly to the mitochondrial gene sequences showing homology to glucocorticoid responsive elements, was performed (Fig. 4).

The result of the ChIP analysis revealed binding of the mitochondrial GR mainly to the regulatory D-loop region of the mitochondrial genome. Other less prominent binding sites were found in the 12 S and NADH I region of the mitochondrial genome. The immunoprecipitated DNA by ChIP analysis was detected and quantified either by semi-quantitative PCR (Fig. 4A) or real-time PCR (Fig. 4B, C).

3.5. Induction of COX I protein synthesis by the mitochondrial GR

The glucocorticoid induced activation of the mitochondrial transcription by the mitochondrial glucocorticoid receptor prompted as to examine whether the increased mitochondrial receptor localization also affects OXPHOS enzyme biosynthesis. For that

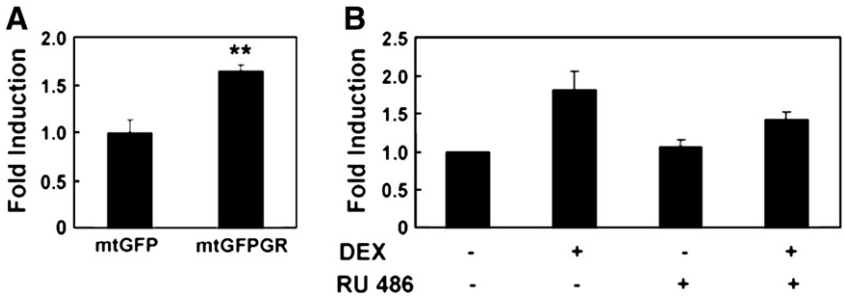

Fig. 3. Mitochondrial RNA synthesis in an organello system. A. Mitochondrial RNA synthesis was measured in equal amounts of isolated mitochondria from HepG2-mtGFP and HepG2-mtGFPGR cells as described in material and methods. Mitochondrial RNA synthesis was evaluated by measuring [α-^{32}P]UTP incorporation into synthesized RNA B. Mitochondrial RNA synthesis was measured in equal amounts of isolated mitochondria from HepG2-mtGFPGR cells in the presence or absence of 10^{-6} M DEX and/or 10^{-5} M RU486. Results are expressed as fold induction of mitochondrial transcription relative compared to A) HepG2-mtGFP cells, B) untreated HepG2-mtGFPGR cells and represent the mean ± SD of at least three individual experiments. $^{*}P<0.05$.

purpose COX I protein levels were evaluated in HepG2-mtGFP and -mtGFPGR cells, following stimulation of the cells either with DEX or ethanol at a final concentration of 10^{-6} M and 0.1%, respectively, for 6 h. β-actin was used for the normalization of the results. As shown in Fig. 5, overexpression of mitochondrial GR caused increase in COX I protein synthesis in both cases examined.

3.6. Effect of mitochondrial GR on mitochondrial ATP content

Mitochondrial GR translocation should be closely associated with the energy status of the cell and may constitute a regulatory mechanism of the cell to face its energy demands. In our model system, the activation of the mitochondrial transcription and OXPHOS enzyme biosynthesis by the stable overexpression of mtGR may also lead to increase in mitochondrial capacity to produce ATP. To test this hypothesis, mitochondrial ATP level of HepG2-mtGFP and HepG2-mtGFPGR cells, preincubated or not with 10^{-7} M DEX for 16–20 h, was measured as described in material and methods. HepG2-mtGFPGR cells showed an approximately 1.5–2 fold increase in ATP level compared to control cells. DEX causes an approximately 20% increase in mitochondrial ATP content in HepG2-mtGFP cells, whereas ATP level in HepG2-mtGFPGR cells is not significantly affected by DEX. Preincubation of cells with 0.2 μM rotenone for 16–20 h in the presence or absence of DEX caused elimination in mitochondrial ATP production in both cell lines examined (Fig. 6).

4. Discussion

The mode of action of glucocorticoids on target cells has been a field of intense experimentation, following the seminal observation that steroid hormones act by way of gene activation [review in 10,12,24]. The induction of gluconeogenic enzymes in the rodent liver was one of the early models to test the hormone–gene activation hypothesis and was instrumental, among other models, to validate the hypothesis [2–6]. An early intriguing finding was the massive induction of RNA synthesis by glucocorticoids in the liver cells, encompassing all types of RNA-HnRNA, ribosomal RNA, t-RNA, most of which was degraded in the nucleus. Importantly, it has been

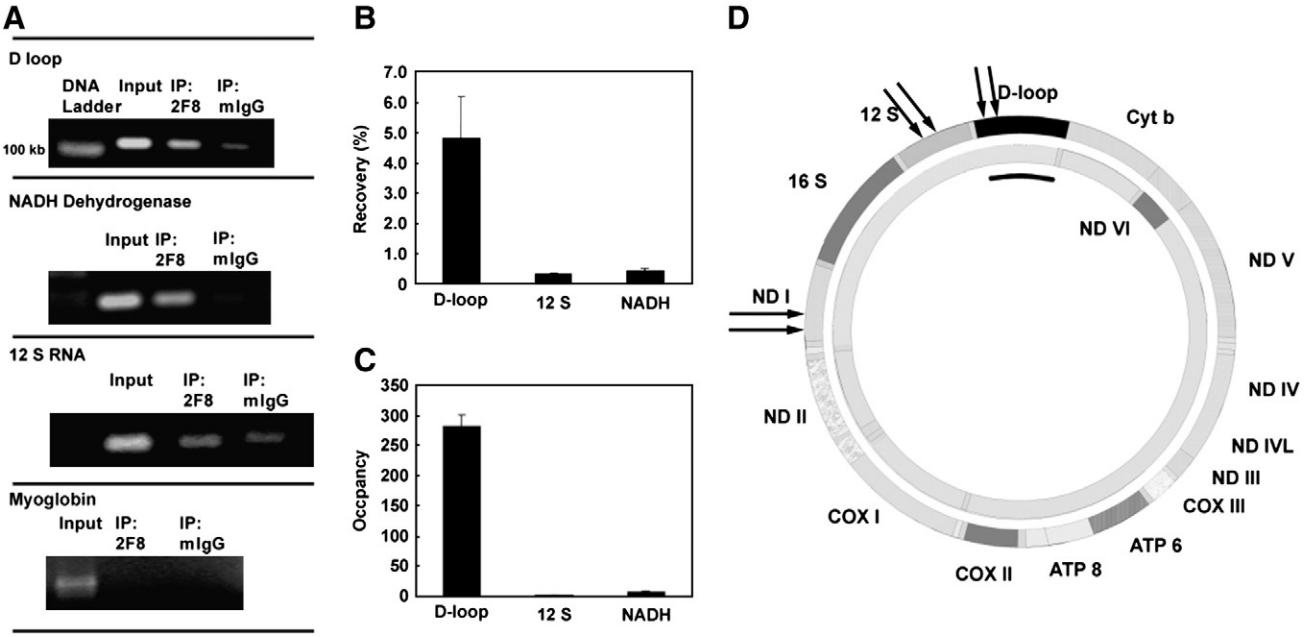

Fig. 4. Binding of the glucocorticoid receptor to the D-loop of the mitochondrial DNA of HepG2 cells. HepG2 cells were subjected to a ChIP assay using specific antibodies against GR (2F8) or nonspecific IgG as described in Materials and methods. Levels of immunoprecipitated DNA encompassing the putative hormone response elements located in the mitochondrial D-loop, the NADH dehydrogenase I gene and the 12 S gene were assessed either by semiquantative PCR (A) or real-time PCR (B, C). 10% input was used. Myoglobin gene was used as negative control. The results of three independent experiments as means ± S.D. are shown in B, C and represent the recovery and occupancy of the mitochondrial DNA sequences. Recovery of DNA was calculated as percentage of input material of the immunoprecipitation. D. The mammalian mitochondrial genome. Sites of primers location are indicated by arrows.

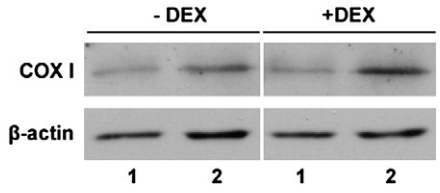

Fig. 5. Activation of COX I protein synthesis in HepG2 cells by the mitochondrial GR. Equal amounts of total protein extracts from HepG2-mtGFP (1) and HepG2-mtGFPGR (2) cells were examined for COX I protein expression applying Western blot analysis. Protein levels of β-αctin were used for the normalization of the results.

observed that mitochondrial RNA is also induced by glucocorticoids [3,6]. Recent results from bioarray analyses reveal many different functional gene classes regulated by glucocorticoids. These functional classes include genes involved in energy metabolism, signal transduction, cell proliferation and differentiation, immune response, transcription, apoptosis, redox regulation, protein transport system, and protein trafficking [7–9]. Although the results from bioarrays can account for part of the induced RNA, the nature of the rapidly turning-over nuclear RNA is still unknown, and could involve the recently discovered various types of regulatory RNA molecules, discussed by various authors [25,26]. The hormonal mobilization of the transcriptional, post transcriptional and translational machinery of the cell is highly energy consuming and indeed, among the proteins induced by glucocorticoids, are enzymes involved in energy production, e.g. OXPHOS, some encoded by the nuclear and some by the mitochondrial genome. How glucocorticoids induce and coordinate transcription of genes in two different cell organelles is generally accepted as the effect of the hormone on nuclear OXPHOS genes and on nuclearly encoded mitochondrial transcription factors, which subsequently enter the mitochondrion to induce mitochondrial DNA transcription. The presence of glucocorticoid receptors in mitochondria and of GRE-like sequences in the mitochondrial genome suggested an additional mode, a direct-effect of the mitochondrially localized receptor in mitochondrial transcription [11,13]. To test this hypothesis HepG2 hepatocarcinoma cells, which stably express a mitochondrial targeted GFPGR or a mitochondrial targeted GFP protein were produced and examined for their efficiency to activate mitochondrial transcription in the presence of DEX and/or

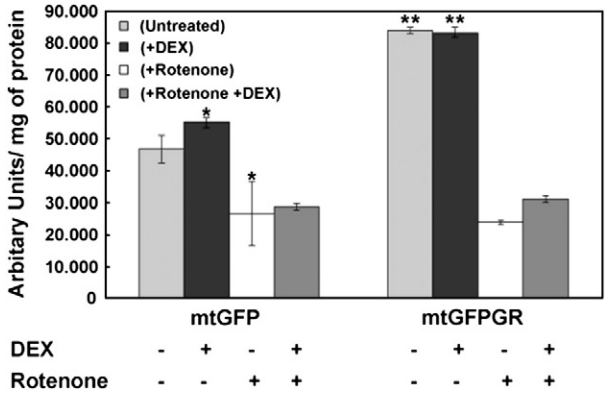

Fig. 6. Effect of mitochondrial GR on mitochondrial ATP level in HepG2 cells. Equal number of HepG2-mtGFP and HepG2-mtGFPGR cells were grown on 15 cm dishes. At 70–80% confluency cells were incubated or not with 0.2 μM rotenone (diluted in DMSO) in the absence or presence of 10^{-7} M DEX (diluted in EtOH), for 16 h, at 37 °C. Control cells were incubated with equal volumes of DMSO and/or EtOH, respectively. Subsequently cells were washed with PBS, harvested, homogenized, and subjected to crude mitochondrial isolation as described previously [17]. Mitochondrial ATP content of isolated mitochondria was measured by bioluminescence assay as described in material and methods. Results are expressed as arbitrary units of luciferase activity/mg of mitochondrial protein and represent the mean ± SD of at least three independent experiments. $^{*}P<0.05$ compared to HepG2-mtGFP untreated cells; $^{**}P<0.001$, compared to similarly treated HepG2-mtGFP cells.

α-amanitin, the well known inhibitor of the DNA dependent RNA polymerase II [20,22,23], which in vivo inhibits not only HnRNA, but also ribosomal and t-RNA synthesis, but does not affect mitochondrial transcription [20,22]. Both non-transfected and stably transfected cells reacted to the addition of dexamethasone with an increased mitochondrial transcription. Among the genes induced were the genes encoding the mitochondrial ribosomal 12 S and 16 S RNA, and the mitochondrial encoded OXPHOS genes ND I, ND II, ND III, ND IV, Cyt b, ATP 6, ATP 8, and COX I. In HepG2 cells DEX also caused induction of the mitochondrial transcription factors TFAM, TFB1M, and TFB2M.

As was expected, in the presence of α-amanitin; the dexamethasone induced expression of the nuclearly encoded mitochondrial transcription factors genes TFAM, TFB1M, and TFB2M was inhibited. The important finding was that in spite of the inhibition of the nuclearly encoded transcription factors, mitochondrial gene transcription although occasionally attenuated, still was induced to a level of 70–100% in respect to cells not treated with the α-amanitin. This leads us to conclude that certainly there is a nuclear contribution to the dexamethasone mitochondrial transcription induction, but that dexamethasone can also affect directly the mitochondrial transcription apparatus by way of the mitochondrial GR. This is also supported by the finding of a direct GR-mitochondrial DNA interaction, specifically on the regulatory D-loop site, which was identified in previous publications as a potential GR binding site on the basis of its sequence homology to GREs, by gel retardation assays and by transfection experiments [27–29].

The hypothesis of a direct effect of GR on mitochondrial transcription with a mode of action similar to that of the nuclear receptor is further supported by our observations showing increased RNA synthesis in a mitochondrial organello system of HepG2-mtGFPGR cells in the presence of DEX and attenuation of this induction in the presence of the GR antagonist, RU486. Moreover, the observed co-immunoprecipitation of the endogenous mtGR with the mtGFPGR by the GFP antibodies may indicates the existence of GR-mtGFPGR interactions in the mitochondrial environment of HepG2-mtGFPGR cells. Experiments are underway to identify mitochondrial GR partners, and other regulatory molecules that participate in the GR-induced activation of the mitochondrial transcription. GR molecules and their interacting partners may constitute regulatory components of the mitochondrial nucleoid structure that contains mtDNA and DNA binding proteins and is well established that exert crucial role in mtDNA replication and transcription [30,31]. Recently it is proposed that nucleoids may play an even broader role in mitochondrial biogenesis [31], a process that is also suggested to be regulated by streroid and thyroid hormones [10,32–34].

The activation of the mitochondrial mRNA synthesis by increased expression of the mitochondrially localized glucocorticoid receptor may also lead to activation of OXPHOS enzyme biosynthesis, as indicated by our results showing increased COX I protein synthesis in HepG2-mtGFPGR cells, and subsequently to increase in mitochondrial ATP content, as was observed in our experimental model. DEX did not significantly affect the capacity of mitochondria to produce ATP in HepG2-mtGFPGR cells. This effect may suggest the existence of regulatory and feedback mechanisms that control and suppress the excess OXPHOS enzyme biosynthesis and activation. In this context, changes in intramitochondrial ATP levels have been proposed as a regulatory mechanism for mitochondrial mRNA synthesis and OXPHOS enzyme activation [14,35]. The elimination in ATP content in the presence of rotenone, an inhibitor of respiratory chain, verified the mitochondrial origin of the GR-induced increase in ATP production.

In addition to GR, other steroid and thyroid receptors have been detected in mitochondria and important efforts have been devoted towards elucidating the role of these receptors in mitochondria [10,36,37]. A specific type of mitochondrial thyroid receptor was found by Casas et al. [16] and a direct effect of T3 on mitochondrial

transcription in an in organello mitochondrial transcription system has been shown establishing the role of the mitochondrial thyroid receptor [14,15,32,33,38]. Estrogen and androgen, receptors have also been found in mitochondria of several types of cells and their involvement in the regulation of mitochondrial functions has been proposed [reviewed in 13,36–40]. Recently the involvement of the mitochondrial glucocorticoid receptor in the regulation of the glucocorticoid-induced apoptosis and modulation of cellular plasticity has been suggested [41,42].

The present work gives the first insights into the molecular mechanisms underlying the GR-mediated mitochondrial transcription providing evidence for a direct involvement of the mitochondrially localized glucocorticoid receptor in the regulation of mitochondrial transcription and OXPHOS enzyme biosynthesis by binding to mtDNA and activation of mitochondrial gene expression. Moreover, results from this study prove that this action takes place in addition to a nuclear action of glucocorticoids on mitochondrial genes by way of induction of genes of the nuclearly encoded mitochondrial transcription factors, which then enter mitochondria to regulate mtDNA transcription and OXPHOS enzyme biosynthesis.

Recently, in addition to nuclear receptors, the mitochondrial localization of several other transcription factors with nuclear actions including NF-κB, p53, AP-1, STAT-3 but with increasing importance for mitochondrial functions has been suggested [36,43–45]. The mitochondrial localization of these molecules potentiates their regulatory significance and renders them coordinators of nuclear and mitochondrial actions in important functions, among them energy production, mitochondrial dependent apoptosis, oncogenic transformation and immune responses [36,39–45].

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