Binding of mitochondrial and nuclear proteins from mouse and human cells to GRE-like elements of mouse mitochondrial DNA

C. TSIRIYOTIS[1], D.A. SPANDIDOS[1,2] and C.E. SEKERIS[1,3]

[1]Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, Athens 116 35, Greece; [2]Medical School, University of Crete, Heraklion, Crete; [3]Department of Biology, University of Athens, Athens, Greece

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Abstract. In the context of the hypothesis that the mitochondria represent primary sites of steroid hormone action we have evaluated the possible role of two nucleotide sequences partially homologous to Glucocorticoid Responsive Elements (GREs) present in the cytochrome oxidase subunit I gene of mouse and human mitochondrial DNA, as binding sites for mitochondrial and nuclear regulatory protein(s). Using a gel retardation assay, we examined whether proteins contained in mitochondrial and nuclear extracts from untreated and dexamethasone treated MCF7 and LATK$^-$ cell lines bind to these oligonucleotides. Both the mitochondrial and nuclear extracts from these cell lines contain protein(s) that bind to the oligonucleotides, as well as to the GRE of the human metallothionein II_A promoter, binding which was considerably enhanced when the extracts were derived from cells previously treated with dexamethasone.

Introduction

Steroid hormones regulate metabolic and developmental processes by interacting with receptor proteins, representing ligand-dependent nuclear transcription factors, subsequent binding of the hormone-receptor complex to enhancer elements, called hormone responsive elements (HRE) and modulation of gene expression (1-3). The presence of HREs in genes implies potential hormone responsiveness of these genes.

We have postulated, based, among other findings (4-7), on the demonstration of the presence in human and rodent mitochondrial DNA of nucleotide sequences partially homologous to HREs, a direct action of steroid hormones on mitochondrial gene expression (8,9). The presence of glucocorticoid receptor in mitochondrial extracts of liver in dexamethasone treated rats, has been demonstrated both by electron microscopy and Western blotting (10; Sekeris CE, Demonakos C, Markovic-Djordjevic R, Papalopoulou M, Spandidos DA, Tsawdaroglou N and Tsiriyotis C, unpublished results) providing further support to this hypothesis.

In this paper we have evaluated, by a gel retardation assay, the role of two nucleotide sequences present in the cytochrome oxidase subunit I gene, showing partial homology to GREs, as binding sites for regulatory proteins present in mitochondrial and nuclear extracts from two cell lines, one human and one mouse, subjected or not, to inducing dose of dexamethasone. As is well established both cell lines possess glucocorticoid receptors (11,12) and thus are responsive to glucocorticoid treatment.

To this aim we have synthesized oligonucleotides containing the two putative mitochondrial GREs, as well as an oligonucleotide containing a GRE from the human metallothionein II_A promoter, which, as known, is a binding site for glucocorticoid receptor (13), to be used as reference sequence.

Materials and methods

Two cell lines were used; MCF7 human breast cancer cells and LATK$^-$ mouse fibroblasts. After treatment of the cell lines with dexamethasone, nuclear and mitochondrial extracts were prepared from treated and untreated cells. The extracts have been used in binding experiments with double stranded ^{32}P-labelled synthetic oligonucleotides, containing the putative GREs from the mitochondrial DNA (mtCOIGREI and mtCOIGREII), or a known GRE from the human metallothionein II_A promoter (hMTII$_A$GRE).

Cell culture. Both cell lines were grown in 75cm^2 cell culture flasks in DMEM/F12 1:1 (Gibco) medium, containing 10% fetal calf serum (FCS), in a humidified atmosphere of 95% air and 5% CO_2, at 37°C. 48 hours prior to dexamethasone administration, the medium was replaced with DMEM/F12 1:1, containing 10% FCS inactivated by dextran treated active charcoal. 24 hours after dexamethasone administration the cells were washed with PBS and collected by scraping. The same procedure was followed for control flasks without dexamethasone treatment.

Correspondence to: Professor C.E. Sekeris, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, Athens 116 35, Greece

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Nuclear extract preparation. The cells were homogenized in 2 ml hypotonic buffer (25 mM Tris HCl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF). The nuclei were pelleted at 2500 rpm for 10 min at 4°C, then washed 3 times with 2 ml isotonic buffer (25 mM Tris HCl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM Surcose, 0.5 mM DTT, 1 mM PMSF) and lysed with an extraction buffer (25 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% Triton, 0.5 mM DTT, 0.5 mM PMSF). Nuclear debris was removed by centrifugation at 25000 rpm for 1 hour in a Beckman 80 Ti rotor at 4°C. The protein concentration of the supernatant was measured as described by Bradford (14).

Mitochondrial extract preparation. The cells were lysed in homogenization buffer (10 mM Hepes, 250 mM surcose, 1 mM EDTA, 0.002% NaN_3, 100 μM PMSF) using a glass homogenizer. Mitochondria were purified by ultra-centrifugation with a Beckman 80 Ti fixed angle rotor, at 35,000 rpm, for 1 h at 4°C, using a Percoll (Pharmacia) gradient containing 0.15 M surcose. The purified mitochondria were broken in a salt buffer (20 mM Tris HCl pH 7.5, 0.6M NaCl, 10 mM ß-mercaptoethanol, 10% glycerol, 100 μM PMSF) and the debris was removed by ultra-centrifugation in a 80 Ti Beckman rotor at 40,000 rpm at 4°C for 90 min. Protein estimation was carried out as for nuclear extracts.

Chemical synthesis of oligonucleotides. The following six single stranded oligonucleotides (27 mers) [1-6] were synthesized on an Applied Biosystems 381A DNA synthesizer:

mtCOIGRE I 6018-6033

5'-AGCTTACCAGCATCTGTTCTGAATTCA-3' [1]
3'-ATGGTCGTAGACAAGACTTAAGTTCGA-5' [2]
GRE Bam HI

mtCOIGREII 6455-6469

5'-AGCTTTTTCCACTATGTTCTGAATTCA-3' [3]
3'-AAAAGGTGATACAAGACTTAAGTTCGA-5' [4]
GRE Bam HI

hMT II_A GRE

5'-AGCTTGGTACACTGTGTCCTGAATTCA-3' [5]
3'-ACCATGTGACACAGGACTTAAGTTCGA-5' [6]
GRE Bam HI

The first [1] oligonucleotide is complementary to the second [2], the third [3] to the fourth [4] and the fifth [5] to the sixth [6]. When annealed, they produce double-stranded GREs. They also contain BamHI restriction sites as indicated and HindIII ends. The number shows their position in the mt genome (in the cytochrome oxidase I gene). The hMT II_A GRE oligonucleotide contains the GRE from the human metallothionein promoter, which is responsive to glucocorticoids. The positions of mtCOIGREI and mtCOIGREII in the mitochondrial genome are indicated in Fig. 1 (8,15).

After synthesis, the oligonucleotides were removed from the column by elution with 3 x 1 ml of ammonia, incubated at 55°C overnight for deprotection and further purified through an oligonucleotide purification cartridge (OPC) from Applied Biosystems. Annealing of complementary couples was carried out by incubation of 0.05 M in TE each strand at 90°C and slow cooling to less than 30°C. To confirm the formation of double stranded oligonucleotides, samples were run on an 8% polyacrylamide gel parallel to the single stranded oligonucleotides. Oligonucleotides containing the NF1 or Sp1 binding sequences were a gift from Dr A. Pintzas.

Oligonucleotide labelling. The three double stranded oligonucleotides were end-labelled with γ^{32}P-ATP using T4 polynucleotide kinase (Boehringer). The reaction was carried out for 30 min at 37°C, then at 70°C for 5 min, at 37°C for 10 min, at room temperature for 5 min and on ice for 5 min.

Gel retardation assays. DNA binding reactions were carried out as follows: 2,000 cpm γ^{32}P-oligonucleotide were mixed with nuclear or mitochondrial proteins (20 μg) in binding buffer (50 mM Hepes pH 8.0, 500 mM NaCl, 0.5 mM PMSF, 0.5 mg/ml BSA, 20 % glycerol, 1 mM EDTA) plus 1 mM DTT and 150 mg/ml poly (dI-dC). The reaction mixture was incubated for 30 min at 4°C. For the competition experiments, an incubation of 20 min at 4°C, in the presence of the competitor, was carried out, prior to the addition of the radioactive probe. Samples were electrophoresed on 5% polyacrylamide gels, then dried and exposed to X-ray film (RX Fuji) at -70°C.

Results

LATK$^-$ cells were cultured in medium containing 10% charcoal inactivated serum for 48 hours and then either treated with 1 μM dexamethasone for 24 h, or left untreated. From treated and untreated cells nuclear and mitochondrial extracts were prepared. All extracts were used in retardation assays involving the three synthetic oligonucleotides mentioned in Materials and methods, in order to investigate the presence of protein(s), among them possibly the glucocorticoid receptor, which bind to the oligonucleotides and whether this binding is influenced by glucocorticoid treatment. The same experiments were carried out with the MCF7 cell line.

In the following two figures are presented the results of the gel retardation assays in the presence of mitochondrial (Fig. 2) and nuclear (Fig. 3) extracts, respectively, of LATK$^-$ cells, not subjected to dexamethasone treatment (Fig. 2, lanes 1, 6 and 11 and Fig. 3, lanes 2, 4, 6, 8, 10 and 12). Both extracts show the presence of protein(s) binding to all three oligonucleotides used. This binding is increased if the extracts are derived from cells previously treated with an inducing dose (1 μM) of dexamethasone (Fig. 2, lanes 2, 7 and 12 and Fig. 3, lanes 3, 7 and 11). This increase of binding activity after hormonal induction is evident particularly in the mitochondrial extracts.

It is apparent that the same retardation band is observed after interaction of the mitochondrial and nuclear extracts from LATK$^-$ cells, both with mtGREI, and mtGREII and with the hMTII$_A$GRE. We then performed competition experiments using as competitors mtCOIGREI, mtCOIGREII, hMTII$_A$GRE

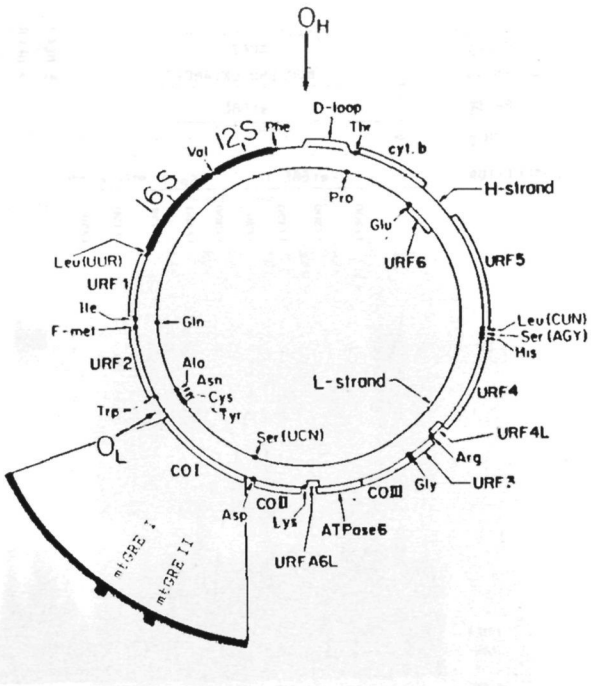

Figure 1. The mitochondrial genome of mammalian cells, indicating the position of GRE-like elements (mtCOIGREI and mtCOIGREII) (taken from references 8 and 14).

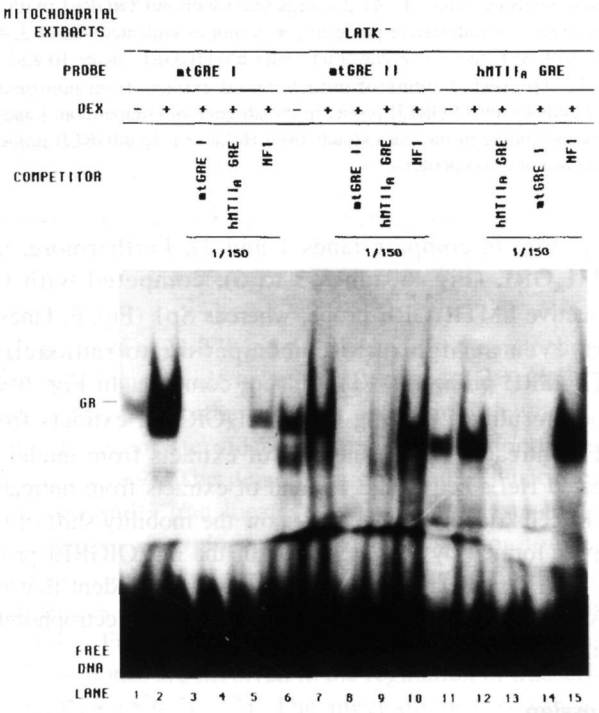

Figure 2. Electrophoretic mobility shift analysis using mtCOIGREI (mtGREI), mtCOIGREII (mtGREII) and hMTII$_A$GRE oligonucleotides and mitochondrial extracts from LATK$^-$ cells not treated and treated with 1 μM dexamethasone. GR indicates the band resulting from protein binding to the probe. Binding to mtCOIGREI, lanes 1-5, to mtCOIGREII, lanes 6-10 and to hMTII$_A$GRE, lanes 11-15. Non-treated cells, lanes 1, 6 and 11, dexamethasone treated, lanes 2, 3, 4 ,5, 7, 8, 9, 12, 13, 14 and 15. Competition with mtCOIGREI (mtGREI), lanes 3, 8 and 13, with mtCOIGREII (mtGREII), lanes 4, 9 and 14, with hMTII$_A$GRE, lanes 4, 9 and 13 and with NF1, lanes 5, 10, 15.

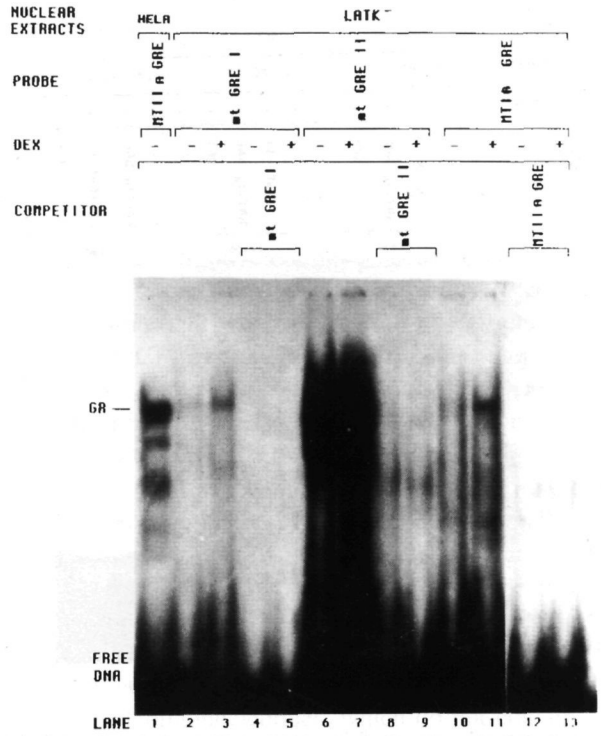

Figure 3. Electrophoretic mobility shift analysis using mtCOIGREI (mtGREI), mtCOIGREII (mtGREII) and hMTII$_A$GRE oligonucleotides and mitochondrial extracts from LATK$^-$ cells not treated and treated with 1 μM dexamethasone. GR indicates the band resulting from protein binding to the probe. Binding to mtCOIGREI, lanes 2-5, to mtCOIGREII, lanes 6-9 and to hMTII$_A$GRE, lanes 11-13. Non-treated cells, lanes 2, 4, 6, 8, 10 and 12, dexamethasone treated, lanes 3, 5, 7, 9, 11 and 13. Competition with mtCOIGREI, lanes 4 and 5, with mtCOIGREII, lanes 8 and 9 and with hMTII$_A$GRE, lanes 12 and 13.

and NF1, the latter containing no sequences related to GREs. mtCOIGREI (Fig. 2, lanes 3 and 14), mtCOIGREII (Fig. 2, lane 8) and hMTII$_A$GRE (Fig. 2, lanes 4, 9 and 13) competed binding of mitochondrial proteins to mtCOIGREI, mtCOIGREII and hMTII$_A$GRE. On the other hand, NF1 showed only partial competition with mtGREI (Fig 2. lane 5) and no competition with mtCOIGREII (Fig. 2, lane 10) and hMTII$_A$GREII (Fig. 2, lane 15). Similar competition experiments were carried out with nuclear extracts from LATK$^-$ cells. All three oligonucleotide probes used (mtCOIGREI, mtCOIGREII and hMTII$_A$GRE) competed effectively (Fig. 3, lanes 4, 5, 8, 9, 12 and 13). Nuclear extracts from HeLa cells show binding to hMTII$_A$GRE, exhibiting the same electrophoretic mobility shift (Fig. 3, lane 1) as the extracts from LATK$^-$ cells.

Similar experiments to those as with LATK$^-$ cells were carried out using mitochondrial and nuclear extracts from MCF7 cells (Figs. 4, 5 and 6). As in the experiments with LATK$^-$ cells we were able to detect the presence of proteins binding to mtCOIGREI (Fig. 4, lanes 1-5), mtCOIGREII (Fig. 4, lanes 6-10) and hMTII$_A$GRE (Fig. 4, lanes 11-15) in mitochondrial extracts from control and dexamethasone treated MCF7 cells. Binding to the three probes was significantly elevated in the mitochondrial extracts compared to dexamethasone treated cells (Fig. 4, compare lanes 1 and

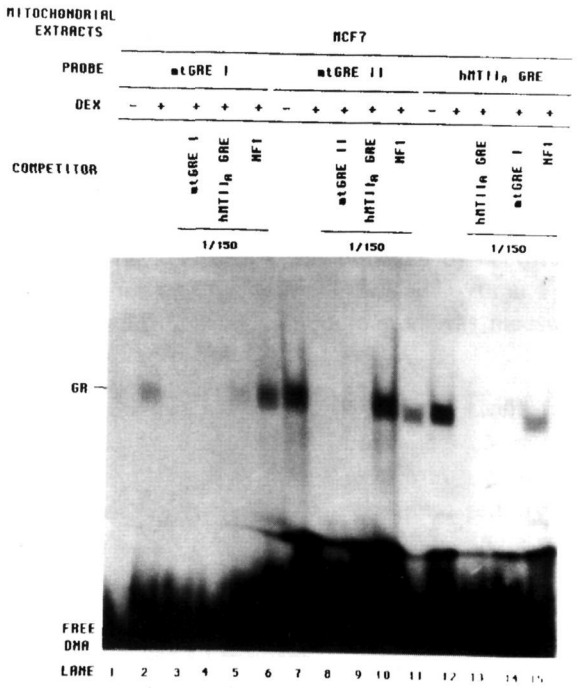

Figure 4. Electrophoretic mobility shift analysis using mtCOIGREI (mtGREI), mtCOIGREII (mtGREII) and hMTII$_A$GRE oligonucleotides and mitochondrial extracts from MCF7 cells not treated and treated with 1 μM dexamethasone. Binding to mtGREI, lanes 1-5, to mtCOIGREII, lanes 6-10 and to hMTII$_A$GRE lanes 11-15. Non-treated cells, lanes 1, 6 and 11, dexamethasone treated, lanes 2, 3, 4, 5, 7, 8, 9, 12, 13, 14 and 15. Competition with mtCOIGREI, lanes 3 and 14, with mtCOIGREII, lane 8 with hMTII$_A$GRE lanes 4, 9 and 13 and with NF1, lanes 5, 10 and 15.

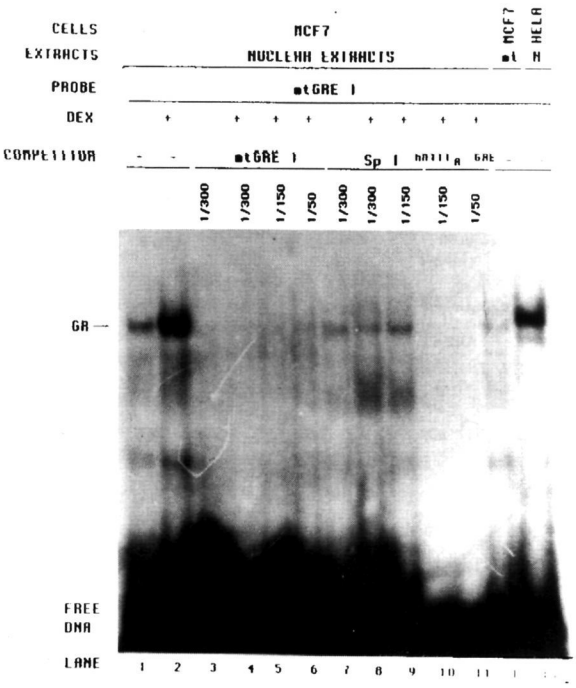

Figure 5. Electrophoretic mobility shift analysis using mtCOIGREI (mtGREI), mtCOIGREII (mtGREII) and hMTII$_A$GRE oligonucleotides and mitochondrial extracts from MCF7 cells not treated and treated with 1 μM dexamethasone. Binding to mtCOIGREI probe, lanes 1-11. Non-treated cells, lanes 1, 3 and 7, dexamethasone treated, lanes 2, 4, 5, 6, 8, 9, 10 and 11. Non-competed, lanes 1 and 2, competition with mtCOIGREI (with the indicated ratio of radioactive probe to competitor as indicated), lanes 3, 4, 5 and 6, with Sp1, lanes 7, 8 and 9 and with hMTII$_A$GRE, lanes 10 and 11. Lane 12 represents binding of mitochondrial extracts from non-treated MCF7 cells to mtCOIGREII probe, in the absence of competition. Lane 13 represents binding of nuclear extracts from HeLa cells to mtGREII probe in the absence of a competitor.

2, 6 and 7 and 11 and 12). Competition experiments showed that, whereas binding to mtCOIGREI was competed with mtCOIGREI or hMTII$_A$GRE (Fig. 4, lanes 3 and 4), binding to mtCOIGREII was competed by mtCOIGREII and hMTII$_A$GRE (Fig. 4, lanes 8 and 9) and binding to hMTII$_A$GRE was competed by hMTII$_A$GRE and mtCOIGREI (Fig. 4, lanes 13 and 14), NF1 did not compete with any of the radioactive probes (Fig. 4, lanes 5, 10 and 15).

In a similar series of experiments using nuclear extracts from control (Fig. 5, lanes 1, 3 and 7) and dexamethasone treated (Fig. 5, lanes 2, 4, 5, 6, 8, 9, 10, 11) MCF7 cells, we were able to demonstrate that binding to mtGREI increased after dexamethasone treatment (Fig. 5, compare lanes 1 and 2). Both mtCOIGREI (Fig. 5, lanes 3, 4, 5 and 6) and hMTII$_A$GRE (Fig. 5, lanes 10 and 11) competed with the mtCOIGREI probe. On the other hand, Sp1 (Fig. 5, lanes 7, 8 and 9), which shows no similarity to GREs, did not compete even at high ratio of competitor to radioactive probe (300 to 1). We include in Fig. 5 the results of binding of mitochondrial extracts from untreated MCF7 cells (Fig. 5, lane 12) and from nuclear extracts of untreated HeLa cells (Fig. 5, lane 13) to mtCOIGREI, so as to show that the formed complex shows the same mobility as the DNA/protein complex formed by the nuclear extracts from MCF7 cells.

The same experiments as above were repeated with nuclear extracts from MCF7 cells using as probe the hMTII$_A$GRE (Fig. 6). Nuclear extracts from dexamethasome treated cells showed increased binding to the hMTII$_A$GRE probe (Fig. 6, compare lanes 1 and 2). Furthermore, the hMTII$_A$GRE (Fig. 6, lanes 3 to 6), competed with the radioactive hMTII$_A$GRE probe, whereas Sp1 (Fig. 6, lanes 7 to 9), even at high ratio of competitor to radioactive hMTII$_A$GRE probe (300/1), did not compete. In Fig. 6 we include results of binding to hMTII$_A$GRE of extracts from MCF7 untreated cells (lane 10), of extracts from nuclei of untreated HeLa cells (lane 11) and of extracts from untreated LATK$^-$ cells (lane 13). We also show the mobility shift of the complex formed by the interaction of the mtCOIGREI probe with MCF7 nuclear extracts (lane 12). It is evident that the DNA/protein complexes formed have the same electrophoretic mobility (compare lanes 1, 2, 10, 11, 12 and 13).

Discussion

Steroid hormones are known effectors of mitochondrial functions (5-7,16-21). It is generally assumed that the steroidal effects on mitochondria are secondary, due to a primary action on nuclear gene expression, by way of the now well understood mechanism of hormone-receptor complex binding to the appropriate Hormone Responsive Elements (HREs). The rapid entrance of glucocorticoids in liver mitochondria (4,5), the evoked stimulation of mitochondrial transcription by glucocorticoids (5,6,23) and

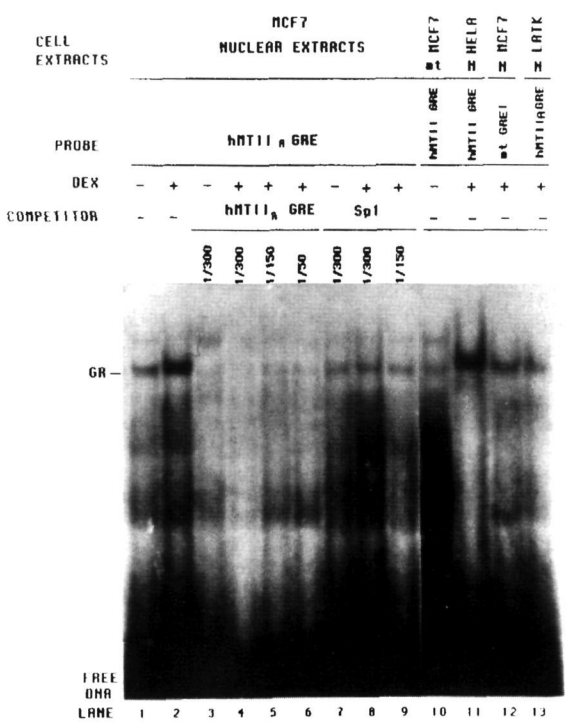

Figure 6. Electrophoretic mobility shift analysis using $hMTII_AGRE$ oligonucleotide and nuclear extracts from MCF7 cells not treated and treated with 1 μM dexamethasone. Binding to $hMTII_AGRE$ probe, lanes 1-9. Non-treated cells, lanes 1, 3 and 7, dexamethasone treated, lanes 2, 4, 5, 6, 8 and 9. Non-competed, lanes 1 and 2, competed with $hMTII_AGRE$, lanes 3, 4, 5 and 6 and with Sp1, lanes 7, 8 and 9. Lane 10 represents binding of mitochondrial extracts of non-treated MCF7 cells to $hMTII_AGRE$ in the absence of a competitor. Lane 11 represents binding of nuclear extracts of dexamethasone treated HeLa cells to $hMTII_AGRE$ in the absence of a competitor. Lane 12 represents binding of nuclear extracts of dexamethasone treated MCF7 cells to mtCOIGREI in the absence of a competitor and lane 13 represents binding of nuclear extracts of dexamethasone treated $LATK^-$ cells to $hMTII_AGRE$ in the absence of a competitor.

other hormones (23-27) and the presence in the mitochondrial genome of nucleotide sequences with partial homology to HREs (8,9), led to the formulation of the hypothesis of a direct action of steroid hormones on mitochondrial gene expression (8,9). The demonstration that the glucocorticoid receptor rapidly translocates from the cytoplasm into liver mitochondria after glucocorticoid administration to rats (10; Sekeris *et al* in preparation) is further support for the hypothesis of a direct mitochondrial effect of steroid hormones. Furthermore, various reports strongly suggest that these hormones are involved in the regulation of transcription of mtDNA (5,6,24-27). The mechanism through which glucocorticoids interact with mitochondria is not yet known. As far as the nucleus is concerned, steroids exert many of their important actions by activating the corresponding receptor, which, by binding to the appropriate HRE, modulates gene expression (5,7). Other steroid effects could be mediated by non-DNA binding mechanisms, involving protein-protein interaction (28,29). Either mechanisms could be applicable in the case of mitochondria, or another novel and distinct mechanism could exist. Our results favour the direct interaction of the receptor with mitochondrial DNA. We have shown that two of the putative mtCOIGREs existing in the cytochrome oxidase subunit I gene interact with protein(s), which also bind to the metallothionein II_A promoter GRE, used as a standard glucocorticoid binding nucleotide sequence. These protein(s) are present in both nuclear and mitochondrial extracts, as both extracts give the same band in the gel retardation assays. Moreover, after dexamethasone treatment, binding of the protein(s) to all three synthetic oligonucleotides is considerably enhanced. We interpret this increase in binding as a result of a dexamethasone induced translocation of the glucocorticoid receptor of MCF7 and $LATK^-$ cells from the cytoplasm to the mitochondria and nuclei. The similarity of the bands formed in the gel retardation assays after interaction of nuclear extracts with the $hMTII_AGRE$ and those formed in the assays using nuclear and mitochondrial extracts with mtGREI and mtGREII strongly suggest that the glucocorticoid receptor represents a major protein in the extracts interacting with the oligonucleotide probes. The binding is quite specific, as the formed DNA/protein bands disappear when the oligonucleotides compete with themselves (or with the metallothionein GRE), but not in competition assays with Sp1 and NF1 oligonucleotides, which do not contain GREs. Similar results in gel retardation assays as those presented in this paper have been obtained using cloned mitochondrial DNA fragments, containing the putative GREs and purified glucocorticoid receptor or rat liver mitochondrial extracts demonstrating binding of GR to the putative mitochondrial GREs (Demonakos C, Tsawdaroglou N and Sekeris CE, unpublished results).

The role that these potential mtGREs may play in the regulation of mitochondrial gene expression is under consideration. The circular mtDNA shows differences both in structure and expression as compared to the nuclear genome. There are no introns in the mitochondrial genome and the genes are transcribed in a way reminiscent of bacterial operons. The two putative mtCOIGREs do not reside in the D-loop area, which contains the promoters and is not transcribed, but in various open reading frames. Do these mtCOIGREs play a role in the activation or enhancement of the transcription, or is their function different from their nuclear counterparts, i.e. could they be involved in the elongation or stability of the nascent transcripts? These questions are currently being explored, using among others, CAT-transfection studies, in which competent cells are transfected with constructs made up of the CAT gene linked to the putative mitochondrial GREs.

The demonstration that mitochondrial and nuclear proteins, probably among them the glucocorticoid receptor, bind to mitochondrial DNA sequences with strong homology to GREs, strongly suggests that the known glucocorticoid effects on both nuclear and mitochondrial metabolism are coordinated by the presence in the mitochondrial and nuclear genome of common regulatory elements.

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