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The Mitochondrion as a Primary Site of Action of Glucocorticoids: the Interaction of the Glucocorticoid Receptor with Mitochondrial DNA Sequences Showing Partial Similarity to the Nuclear Glucocorticoid Responsive Elements

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Six mitochondrial genome sequences, showing strong similarity to the glucocorticoid responsive element consensus sequence (GRE), four localized within the cytochrome *c* oxidase (COX) subunit I and III genes (GREs I–IV) and two within the D-loop region (GREs a and b) have been examined as binding sites of glucocorticoid receptor (GR) from rat liver cytosol. Purified GR from rat liver cytosol binds with high specificity to all potential mitochondrial GREs, as shown by filter retention and gel shift assays. Specific binding of protein(s), present in a mitochondrial extract from dexamethasone-induced mice, to all six putative mitochondrial GREs was also documented by the same methodology. Both purified GR and protein(s) from mitochondrial extract give the same band in the gel retardation assay. Using monospecific anti-glucocorticoid receptor polyclonal antibody (EP), a supershift of the gel retarded protein-DNA band was obtained. These results demonstrate that the mitochondrial genome sequences examined have characteristics of GREs, since they show the capacity to specifically bind the respective receptor protein. These findings support the hypothesis that the mitochondrial genome is a primary site of action of steroid and thyroid hormones (Sekeris C.E.: The mitochondrial genome: a possible primary site of action of steroid hormones, *In vivo* 4 (1990) 317–320).

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INTRODUCTION

Steroid and thyroid hormones have profound effects on the biogenesis and physiological functions of mitochondria in liver and muscle [1–11]. Mitochondrial respiration and oxidative phosphorylation, ion translocation, number and size of mitochondria, known to be influenced by hormone treatment, are of obvious importance in cell metabolism and a number of studies have been concerned with these aspects of mitochondrial functions [4, 5, 12]. Anabolic response of skeletal muscles to androgens is accompanied by a striking increase in the activity of mitochondrial cytochrome *c* oxidase (COX) [7]. The inhibitory effect of progesterone upon mitochondrial respiration is well documented [8, 9]. Also demonstrated is the estrogen-induced accumulation of the mitochondrial mRNA for subunit II of COX in pituitary tumor cells [13]. A very rapid uptake of radiolabelled cortisol in the nuclei and mitochondria of rat liver has been previously demonstrated [1, 14]. Glucocorticoid and thyroid hormones lead to enhanced transcription of the mitochondrial genome, including that of subunit II of COX [1–3,

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Abbreviations: GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; HRE, hormone responsive element; COX, cytochrome *c* oxidase; mtDNA, mitochondrial DNA; TAT, tyrosine aminotransferase; CAT, chloramphenicol acetyltransferase; ctDNA, calf thymus DNA.

15–17]. Very recently it was shown that retinoic acid, whose receptor belongs to the same superfamily of nuclear regulatory transcription factors, as the receptors for steroid and thyroid hormones, can also regulate genes encoding the mitochondrial proteins ATP synthase β, cytochrome *c* I and ND5 [18, 19]. These regulatory agents carry out most of their actions after binding to specific receptor proteins, which then modulate transcription of corresponding genes by binding to the appropriate hormone responsive elements (HREs) [20–22]. It is generally assumed that the steroid and thyroid effects on mitochondria are secondary, due to a primary action on nuclear gene expression, and a mechanism should exist, that coordinates the expression of the nuclear and the mitochondrial genome. A model for the coordination of mitochondrial and nuclear encoded proteins is COX [23–25). In all vertebrates studied so far, this enzyme is composed of 13 subunits with the three largest subunits encoded by the mitochondrial DNA (mtDNA) and the remainder by nuclear genes [26, 27]. Whereas the former subunits are synthesized within the mitochondrial matrix, the latter are translated in cytosolic ribosomes and imported into the organelle [28]. However, the molecular mechanism involved in the coordination of the two genomes, is still not yet clear. Recent findings by Wiesner *et al.* [29], conclude that the nuclear and mitochondrial genomes are regulated by the thyroid hormone not through a common mechanism, but by way of two separate pathways. Taking all available data up to now, the mode of action of those hormones on mitochondrial function has not yet been elucidated. Sekeris and coworkers found the presence of several sequences within the mitochondrial genome showing high similarity to HREs [30, 31]. Considering data mentioned above, he proposed that the mitochondrial genome could be a primary site of action of steroid and thyroid hormones, i.e. the effects of these hormones on the mitochondrial genome could be a consequence of a direct action of hormonal steroids through a mechanism similar to that of the action of steroid hormones on the nuclear genome [30, 31].

If the mitochondrial genome is indeed a primary site of action of glucocorticoids and these hormones exert similar molecular effects, as they do on the nuclear genome, then: (a) the hormone receptor should be present in mitochondria; (b) the receptor should be able to bind specifically to mitochondrial DNA sequences showing similarity to the nuclear GREs; and (c) the result of the interaction of the receptor with the mitochondrial DNA should be a modulation of transcription.

Concerning (a) we have recently reported the presence of GR in liver mitochondria of dexamethasone-induced rats [32]. In this paper we have focused our interest on point (b).

According to Sekeris [30], 8 putative GREs are present in the mouse mitochondrial genome showing varying similarity to the consensus GRE sequence. Three of these are within the COX subunit I gene (positioned at 6018–6032, 6455–6469 and 6709–6722 and denoted GRE I, GRE II and GRE III, respectively), one in the COX subunit III gene (positioned at 9177–9191, denoted GRE IV), two in the D-loop (positioned at 15,779–15,793 and 16,161–16,176, denoted GREa and GREb, respectively), one in the 12S rRNA gene and one in the tRNA-leu gene. The GREs I–IV are 80, 73, 80 and 60% similar, respectively, to the consensus GRE sequence (Fig. 1), and the GREa and GREb 80 and 73%.

In the present study we show, using nitrocellulose filter retention and gel retardation assays, the specific binding of the purified glucocorticoid receptor to mitochondrial DNA restriction fragments containing putative GREs I–IV present within the COX subunit I and III genes and to two chemically synthesized oligonucleotides (24 residues and 22 residues each), representing GREa and GREb, respectively, of the D-loop of the mitochondrial genome. The control band shift experiment was performed by investigating the binding of purified GR to the GRE of MMTV-LTR, which, as known, is a binding site for GR. Similar binding to the six putative GREs was observed using mitochondrial extracts from dexamethasone treated rats, instead of GR. Our results provide strong evidence that the purified GR, as well as the GR present in mitochondrial extracts, specifically bind to all six putative mitochondrial GREs.

EXPERIMENTAL

Materials

Animals. Male Balb C mice, 2 months old, were adrenalectomized 1 day before the experiment and kept on a standard diet and 0.9% saline *ad libitum.* They received 10 μg/100 g body mass of dexamethasone i.p., 10 min before sacrifice.

2–3-month-old male Wistar rats (160–180 g body mass) were adrenalectomized 4 days before sacrifice and kept on a standard diet and 0.9% saline *ad libitum.*

Plasmids. Plasmid MMB25, containing part of the mitochondrial genome (4275–11,167), plasmid MMTV5, containing MMTV-LTR, and 21H plasmid were kind gifts of Dr D. Spandidos (Athens). Plasmid 2 × GRE37TK, containing two GRE sequences of the tyrosine aminotransferase (TAT) regulatory region, was kindly offered by Dr I. Grummt (Heidelberg). Plasmid pBR322 was purchased by Promega.

Chemicals. [γ-^{32}P]ATP (5,000 Ci/mmol) was purchased from Amersham (Bucks, U.K.). DEAE-Sepharose and Percoll were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and Whatman phosphocellulose P-11 was from W & R Balston Ltd (Springfield Mill Maidstone, Kent, U.K.). DNA–cellulose was purchased from Sigma (St Louis, MO, U.S.A.) and restriction enzymes from BioLabs

(England). Nitrocellulose filters 0.45 μm, 6 mm diameter, were purchased from Schleicher and Schuell (Basel, Germany). All other chemicals were of the highest purity grade and were obtained from Sigma (St Louis, MO, U.S.A.).

Methods

Isolation of mice mitochondria. Following the sacrifice of the animals, the livers of mice were perfused with homogenization buffer (10 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.002% NaN_3, 0.1 mM PMSF, 1 mM DTT) and homogenized in 1:3 w/v of the same buffer. The homogenate was centrifuged for 3 min at 3000 rpm in a Beckman J2-21 centrifuge using a JA-20 rotor and the supernatant was subsequently centrifuged for 15 min at 15,000 rpm. The pellet obtained was washed twice with homogenization buffer. The final pellet was resuspended in 22 ml of homogenization buffer, mixed with 23.4 ml of Percoll and 2.6 ml of 2.5 M sucrose and centrifuged for 55 min at 40,000 rpm at 4°C in a Beckman L-8 ultracentrifuge using a Ti-80 rotor. The sharp interface containing the mitochondria was carefully collected and washed further three times in homogenization buffer by centrifugation at 15,000 rpm for 10 min at 4°C. The final pellet was resuspended in 3 ml of buffer (20 mM Tris pH 7.5, 0.6 M NaCl, 10 mM 2-mercaptoethanol, 10% glycerine and 0.1 mM PMSF).

Purification of the glucocorticoid receptor. Partially purified GR from rat liver cytosol was prepared according to the three step purification method described by Wrange *et al.* [33]. The purity of the isolated GR was checked by 9% SDS–PAGE and by Western blotting using polyclonal antibody (EP) against human GR [32]. GR was also partially purified from mice mitochondrial extracts as follows: Isolated mice mitochondria were treated with 600 mM NaCl at 4°C in order to break mitochondrial membranes and were centrifuged at 40,000 rpm 90 min at 4°C in a Beckman L-8 ultracentrifuge using a Ti-50 rotor. The resulting supernatant was diluted 1:10 in a buffer containing 10 mM Tris pH 7.5, 0.1 mM EDTA, 1 mM $MgCl_2$, 0.1 mM DTT and 60 mM KCl (this preparation represents the mice mitochondrial extract) and the extract was further applied on an ion exchange DEAE-Sepharose column (0.8 × 1 cm). The column was eluded with a continuous salt gradient (0–250 mM NaCl in the aforementioned buffer) and 24, 1.25 ml, fractions were collected. Each fraction was examined for protein and salt concentration. The GR is contained in fractions eluded by 120–150 mM salt [33].

Isolation of mitochondrial and MMTV-LTR GREs. The position of the mitochondrial sequences of the six putative GREs are shown in Fig. 1(A). The structure of plasmid MMB25, containing the four putative GREs I–IV and their sequence are shown in Fig. 1(B). The sequence of GREa and b are shown in the next section.

For the investigation of the interaction of mitochondrial GREs I–IV with purified GR and with proteins of the mitochondrial extract by means of the filter binding assay a 3283 bp mitochondrial DNA fragment, containing four putative GREs, originating from the MMB25 plasmid, was cut with NheI and NcoI, isolated from low melting agarose 1% gel electrophoresis and submitted further to SacI and AccI digestion. The resulting fragments were 2100 bp (which does not contain GREs), 813 bp (containing GREII and GREIII), 293 bp (containing GREI), and 166 bp (containing GREIV). In this case the fragments have not been separated on gels and have been used as a mixture.

As control DNA, a 400 bp fragment, originated from pBR322, cut with HinfI and isolated from 1% low melting agarose gel, was used.

In some experiments, as competitor DNA, the 126 bp fragment originated from the 21H plasmid, digested with BamHI and HindIII restriction enzymes and extracted from a 12% polyacrylamide gel, was used.

For the study of the interaction of isolated mitochondrial GREs I and III with purified GR by gel retardation assay, the GREs were isolated as follows: 150 μg of the plasmid MMB25 was treated with restriction enzymes AccI, NcoI and HindIII. The resulting fragments containing mitochondrial GREs (468 bp, GREI; 813 bp, GREII and GREIII; and 77 bp, GREIV), were isolated from low melting agarose 1% gel electrophoresis. After extraction, the fragments were further treated as follows: the 468 bp fragment with AvaII and HinfI resulting in a 40 bp fragment containing GREI; the 813 bp fragment with HpaII, HinfI and Sau3AI resulting in a 126 bp fragment containing GREII and a 34 bp fragment containing GREIII. These fragments were further isolated from 12% polyacrylamide gels according to the procedure described by Sambrook *et al.* [34].

GRE from MMTV-LTR (57 bp fragment) was obtained by submitting plasmid MMTV5 to BamHI and HindIII digestion and subsequently to a 12% polyacrylamide gel electrophoresis.

Chemically synthesized oligonucleotides (GREa and GREb). Single stranded oligonucleotides were chemically synthesized and obtained from the Laboratory of Microchemistry, Institute of Molecular Biology and Biotechnology, Crete. The 24 residue of sense 5′AGCTTAGACATCTGGTTCTTACTG-3′ and the corresponding antisense, contain the nucleotide sequence from the D-loop area of the mitochondrial genome positioned at 15,777–15,796 (underlined) and was denoted GREa, the 22 residue of sense 5′-TCCACTCAAACCCTATGTCCTG-3′ and the corresponding antisense, contain the nucleotide sequence from the D-loop positioned at 16,160–16,175 (underlined) and was denoted GREb. The oligonucleotides were first purified and isolated from 20%

polyacrylamide–urea gels, then annealed for 5 min at 68°C, cooled at room temperature, separated and extracted from 20% polyacrylamide–urea gels. The final concentration of double stranded synthetic oligonucleotides was 25 ng/μl [34].

As specific competitors, in the experiments using chemically synthesized oligonucleotides, a 150 bp fragment and a 400 bp fragment containing 2 GREs were used, both obtained from the 2 × GRE37TK plasmid cut with PstI or PvuII and submitted to 5% polyacrylamide gel electrophoresis. As non-specific competitors, a 59 bp synthetic oligonucleotide (p59) containing part of the sequence of the hsp90 gene was used.

Preparation of monospecific EP polyclonal antibody. An EP, against human GR polyclonal antibody [32] was made monospecific as follows: mice liver cytosol was submitted to a 9% SDS–PAGE and subsequently Western blotted. The 94 kDa band, corresponding to GR, was cut from the nitrocellulose membrane and incubated with EP polyclonal antibody. After incubation, the antibody was reextracted from the nitrocellulose membrane, as previously described [35]. This monospecific antibody was used for the super gel-shift analysis. Proteins of the mitochondrial extract from liver of dexamethasone treated mice (5 μg) were incubated with monospecific EP polyclonal antibody (1:200 ratio of antibody to protein) for 2 h at 4°C before the binding reaction with DNA.

End-labelling of DNA fragments. End-labelling of restriction fragments of mitochondrial DNA, of synthetic oligonucleotides, of MMTV-LTR and of control DNA (400 bp fragment from pBR322) was performed according to Sambrook *et al.* [34], using T_4 polynucleotide kinase and [γ-^{32}P]ATP.

DNA binding. ^{32}P-end-labelled restriction fragments of mitochondrial DNA containing 4 putative GREs (2100 bp, without GRE; 813 bp, GREII and GREIII; 293 bp, GREI; and 166 bp, GREIV; 3 ng), end-labelled restriction fragments of the mitochondrial genome containing 2 putative GREs (40 bp, GREI and 34 bp, GREIII, 10 ng), end-labelled synthetic oligonucleotides containing GREs from the mitochondrial D-loop region (GREa and GREb, 3 ng), end-labelled 57 bp fragment containing GRE from MMTV-LTR (1 ng) and end-labelled control DNA (400 bp fragment of pBR322, 1.2 ng) were incubated with either purified rat liver GR (20–40 ng), or with mitochondrial extract (2–5 μg of protein), for 30 min at 25°C in binding buffer (10 mM Tris pH 7.5, 1 mM $MgCl_2$, 0.1 mM DTT, poly(dIdC)$_2$ and BSA (final protein concentration 3 μg/final volume) in a total volume of 40 μl. The corresponding cpm was between 10,000–40,000. As specific competitors, non-labelled 57 bp fragment containing GRE from MMTV-LTR (7 ng) and 150 and 400 bp fragments containing GREs from the TAT regulatory region of the 2 × GRE37TK plasmid (15 and 10 ng, respectively), were used. As non-specific competitors, calf thymus DNA (100 and 150 ng), a 126 bp fragment of the 21H plasmid (18 ng) and a 59 bp fragment of the p59 synthetic oligonucleotide (18 ng) were used.

Filter retention assay. After the binding reaction the samples were filtered at room temperature through nitrocellulose filters, as previously described [36]. The filters were then washed twice with binding buffer. The protein–DNA complex retained on the filters was eluded in extraction buffer (10 mM Tris–HCl, pH 7.5, 0.14% SDS, 1 mM EDTA, 50 μg/ml yeast tRNA and 100 μg/ml proteinase K) and extracted by phenol. After removing the SDS by adding 100 mM KCl and ethanol precipitation, the recovered radioactivity was measured and the preparations were submitted to 4% polyacrylamide gel electrophoresis [37].

Gel retardation assay. The gel retardation method was applied, as previously described [38], using 4 or 5% polyacrylamide gels in TBE buffer (0.09 M Tris-base, 0.09 M H_3BO_3, 0.0025 M Na_2EDTA). After running the gel under non-denaturing conditions, the gels were dried and the radioactivity was detected by autoradiography at −80°C.

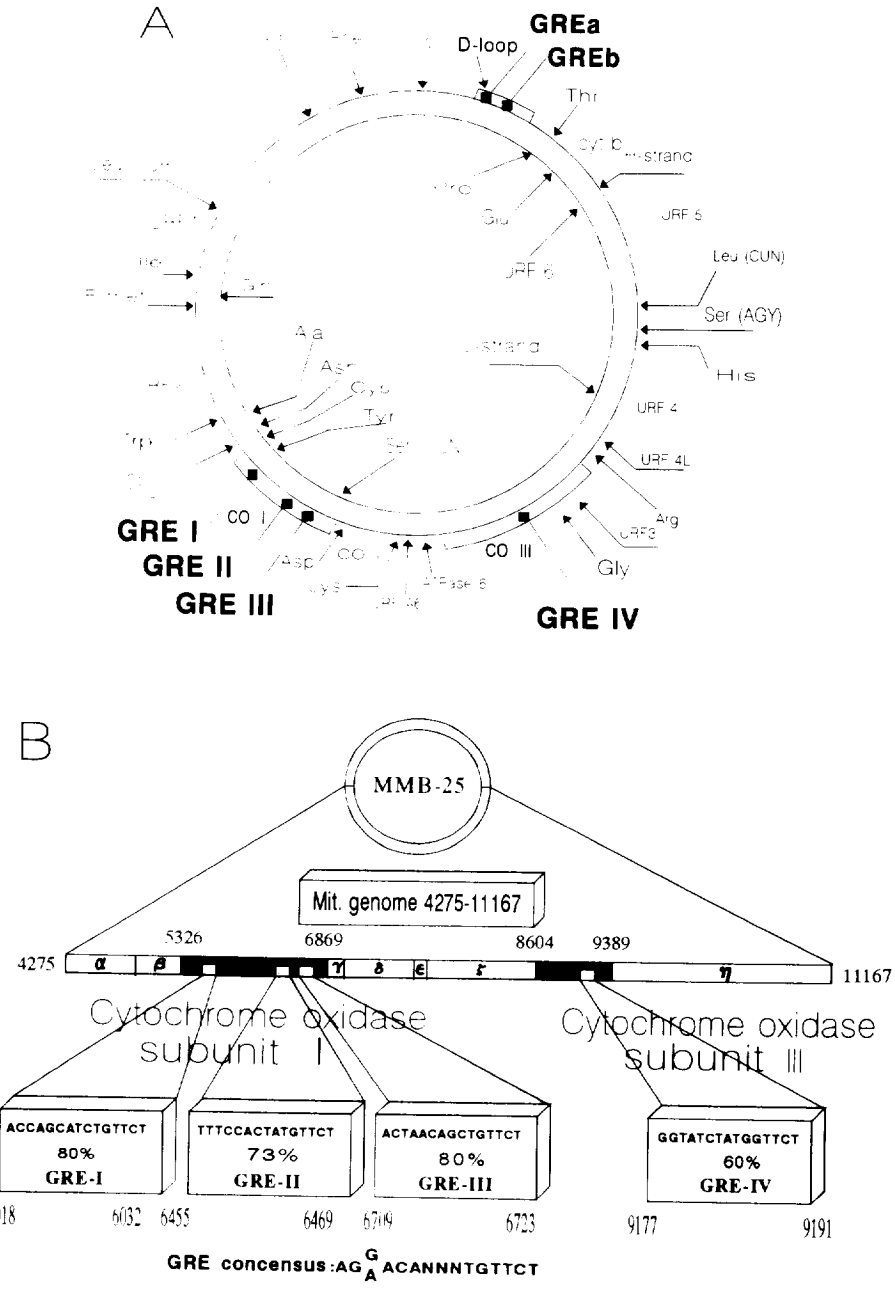
Protein determination. The concentration of proteins was determined according to the method described by Bradford, using the BioRad kit [39].

RESULTS

The interaction of putative GREs I–IV of the mouse mitochondrial genome with GR, using filter retention and gel retardation assays

To demonstrate binding of the GR to the putative GREs I–IV present within the COX I and III genes of the mouse mitochondrial genome [see Fig. 1(A)], two series of experiments were performed, one using filter retention and the second using gel retardation assay. In the first series, using the filter retention assay, a cloned 3285 bp mouse mitochondrial fragment containing the four mitochondrial GREs [Fig. 1(B)], was digested with enzymes AccI and SacI (see Materials and Methods), providing fragments of 2100 bp (containing no GRE), 813 bp (containing GRE II and GRE III), 293 bp (containing GRE I) and 166 bp (containing GRE IV). The fragments were end-labelled with [γ-^{32}P]ATP and incubated with purified GR from rat liver cytosol (Fig. 2). As a control DNA, the ^{32}P end-labelled 400 bp fragment from the pBR322 plasmid was used. Subsequently, the filter retention assay was applied. The complexes retained on the filters, as well as aliquots of the incubation mixture not submitted to filtration, were submitted to polyacrylamide gel electrophoresis (Fig. 2). From Fig. 2 it is evident that the three DNA fragments containing GREs are retained on the filters, leading to the conclusion that the four putative mitochondrial GREs contain binding sites for the GR. The 2100 bp fragment, which does not contain GRE and is not bound on the filter, is not visible on this gel.

Using the same method, we also investigated the ability of protein(s) present in partially purified mitochondrial extracts (see Materials and Methods), obtained from dexamethasone treated mice [32], to bind to the same mitochondrial genome fragments used in the previous experiment, in the presence or absence of calf thymus DNA (100 and 150 ng). We have previously shown that dexamethasone induction of rats leads to a rapid import of GR from liver cytoplasm to the mitochondria [32]. In contrast, the control animals (not treated with dexamethasone) did not show the presence of GR in mitochondria. After performing the binding reaction, the incubation mixtures were filtered through nitrocellulose filters, the filters extracted and



α: tRNA β: tRNA γ: tRNA δ Cytochrome oxidase subunit II ε: tRNA ζ: ATPase 6 η: tRNA

Fig. 1. Schematic representation of the position of GRE-like elements within the mammalian mitochondrial genome. (A) Schematic representation of the mammalian mitochondrial genome, on which the position of six GRE-like sequences, investigated in this paper, is shown. (B) MMB-25 plasmid, containing a fragment of the mouse mitochondrial genome (4275–11,167), showing the position of four putative GREs (GRE I–IV) located within the COX subunits I and III genes and their similarity with the consensus GRE.

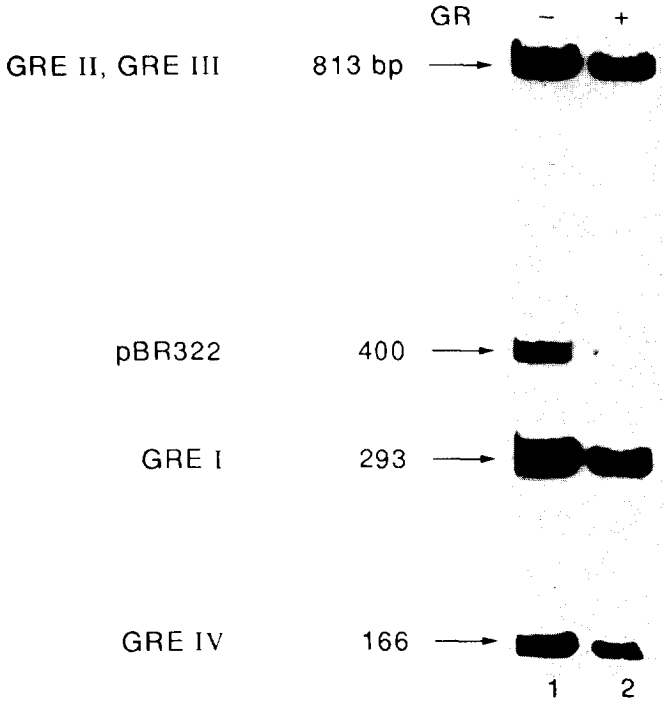


Fig. 2. Polyacrylamide gel electrophoretic analysis of the complexes formed after interaction of rat liver GR and four mitochondrial putative GREs (GRE I–IV) retained on nitrocellulose filters. The mixture of ^{32}P end-labelled fragments of mtDNA originating from the MMB-25 plasmid (2100 bp, no GRE; 813 bp, GRE II and GRE III; 293 bp, GRE I; and 166 bp, GRE IV) (3 ng DNA, 10,000 cpm) and ^{32}P end-labelled control DNA [400 bp fragment from pBR322 plasmid (1.2 ng, 1500 cpm)] were either directly submitted to 4% polyacrylamide gel electrophoresis (lane 1), or were first incubated with rat liver purified GR (40 ng), filtered through nitrocellulose filters, the complexes formed were extracted from the filters and then submitted to 4% polyacrylamide gel electrophoresis (lane 2). Electrophoresis took place under non-denaturing conditions, the gel was dried and autoradiographed at −80°C.

the extracts analyzed by 4% polyacrylamide gel electrophoresis. In parallel, the mixture of the end-labelled GREs was also submitted to electrophoresis (Fig. 3). The mitochondrial extracts obtained from control animals not treated with dexamethasone showed significantly less binding to the four putative GREs (results not shown) which is in agreement with our results reported in ref. [32]. From Fig. 3 it is evident that all three fragments containing four putative GREs show interaction with the mitochondrial proteins and are retained on the filter. In the presence of high concentrations of thymus DNA there is a decrease in the amount of DNA–protein complexes formed, particularly as regards fragments 813 and 166 bp, which is also supported by quantitative analysis of the scanning autoradiography. These results are presented in Table 1.

In the second series of experiments we investigated, by gel retardation assay, the capability for, and specificity of, binding of purified rat liver GR to the mitochondrial GRE fragments isolated from the MMB-25 plasmid. Before performing the gel retardation assay with putative mitochondrial GREs, a control experiment was performed by investigating the binding and the specificity of binding of purified GR to the GREs of the MMTV-LTR, which, as known, are binding sites for GR [40–42]. A 57 bp fragment containing one GRE of MMTV-LTR was obtained from plasmid MMTV5 by BamHI and HindIII cleavage and by extraction from 12% polyacrylamide gel electrophoresis. ^{32}P end-labelled fragment was incubated with the purified rat liver GR in the presence or absence of a specific competitor (a 57 bp fragment of MMTV-LTR) and in the presence or absence of a non-specific competitor (a 126 bp fragment from 21H plasmid) and submitted to a 4% non-denaturing polyacrylamide gel electrophoresis. The results obtained are presented in Fig. 4, showing the specific binding of purified GR to the GRE from MMTV-LTR. For the isolation of the mitochondrial fragments containing GREs we used a different approach. Smaller fragments were thus obtained, a 40 bp fragment containing GRE I, a 126 bp fragment containing GREII and a 34 bp fragment containing GRE III (see Materials and Methods). Due to the stronger similarity of GREI and GREIII to the consensus GRE, as compared to that of GRE II and IV, the following experiment was performed using GREI and GREIII. The fragments were separately isolated from a 12% polyacrylamide gel, ^{32}P end-labelled, incubated with purified rat liver GR under standard conditions and, subsequently, submitted to a 5% non-denaturing polyacrylamide gel electrophoresis. The incubation was carried out in the presence or absence of a specific competitor (a 57 bp fragment of MMTV-LTR containing a GRE) and in the presence or absence of a non-specific competitor (a 126 bp fragment from the 21H plasmid). It is evident from the results presented in Fig. 5, that the purified GR specifically interacts with both isolated mitochondrial GREs I and III, since the binding of the receptor to these fragments is completely prevented with the specific competitor, but not with the non-specific one. These results are in agreement with those obtained using the filter retention method (Fig. 2) and the gel retardation assay (Fig. 4).

The interaction of GREa and GREb of the mouse mitochondrial D-loop region with GR and with mitochondrial extract, using the gel retardation assay

The ability of two synthetically prepared double stranded oligonucleotides, each containing putative GREs from the mitochondrial D-loop region (GREa and GREb), to interact with purified GR and with mitochondrial extracts prepared from liver of dexamethasone treated mice, was tested by gel retardation assays.

The binding of purified GR to GREa and GREb is shown in Fig. 6(A) and (B), respectively. The binding

reaction was carried out under standard conditions, in the absence or the presence of specific competitors (150 and 400 bp fragments containing GRE sequences of the TAT regulatory region), or a non-specific competitor (p59 bp fragment) and samples were subsequently submitted to a 5% non-denaturing polyacrylamide gel electrophoresis. It is evident that the purified GR binds to both GREs from the D-loop mitochondrial DNA region [Fig. 6(A, lane 2) and (B, lane 1)]. There is a partial competition for this with the 150 bp specific competitor, and almost total competition with the 400 bp specific competitor and there is no competition with the p59 bp non specific competitor [Fig. 6(A, lanes 3–5) and (B, lanes 2–4)]. GREa alone shows no radiolabelled band in that region [Fig. 6(A, lane 1)].

Using the same methodology, the binding of GREa and GREb to protein(s) from the mitochondrial extract of dexamethasone induced mice was further investigated (Fig. 7). It is evident that the mitochondrial extract contains protein(s) which bind to both GREs, since in this case the same retardation band is observed as with the binding of purified GR [Fig. 7(A) and (B, lane 1)]. The binding is specific, since in the presence of specific competitors (150 and 400 bp fragments) there is no binding [Fig. 7(A) and (B, lanes 2 and 3)]. The non-specific competitor (p59 bp fragment) does

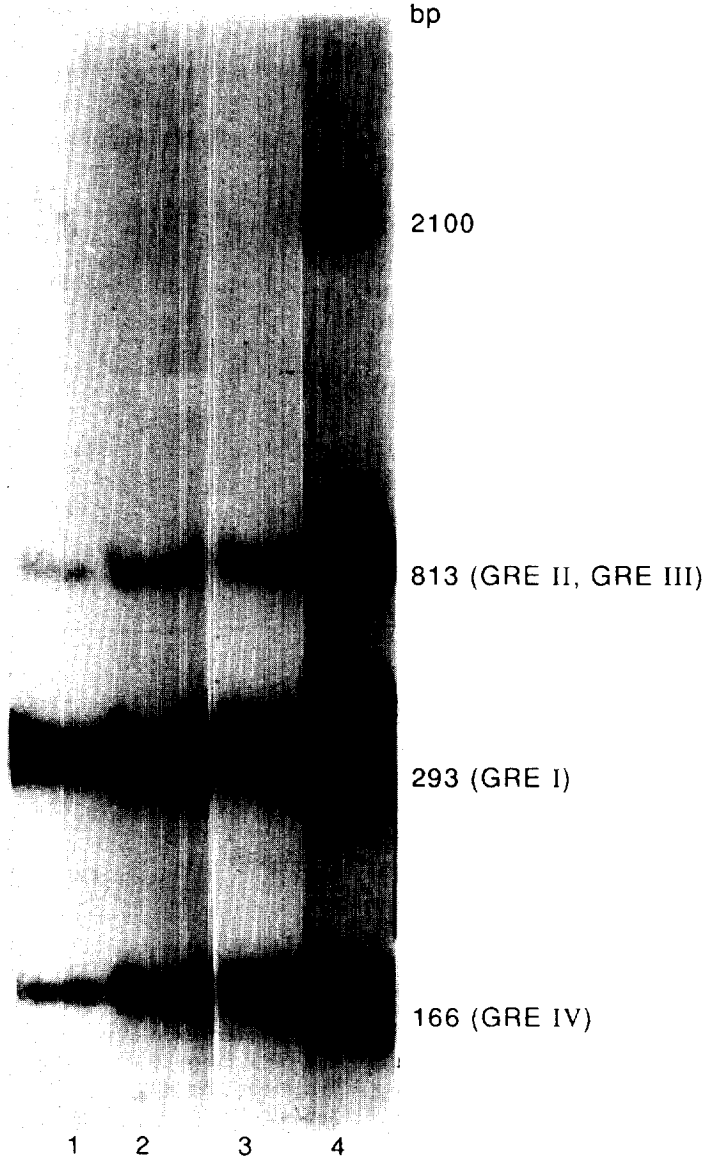


Fig. 3. Polyacrylamide gel electrophoretic analysis of the complexes formed after incubation of mitochondrial extracts with mitochondrial DNA fragments containing four putative GREs (GRE I–IV). The mixture of ^{32}P end-labelled fragments of mtDNA, originating from the MMB-25 plasmid used in the experiment depicted in Fig. 2, was incubated with 2 μg of mitochondrial proteins, in the presence of 150 ng of calf thymus DNA (lane 1), in the presence of 100 ng of calf thymus DNA (lane 2) and in the absence of calf thymus DNA (lane 3), filtered through nitrocellulose filters, the filters extracted and the extracts submitted to 4% polyacrylamide gel electrophoresis and autoradiography. Lane 4 depicts electrophoresis of an aliquot of the incubation mixture not submitted to filtration. Electrophoresis and autoradiography were performed as described in Fig. 2.

Table 1. Quantitative evaluation of the percentage of the binding of mitochondrial proteins to the four putative mitochondrial GREs (GRE I–IV)

DNA fragments	DNA–protein complexes (%) Without competitor	With 100 ng ctDNA	With 150 ng ctDNA
GRE I (293 bp)	100	95	51.1
GRE II, GRE III (813 bp)	100	82	24.5
GRE IV (166 bp)	100	97.6	26.3

Filter retained and extracted GRE–protein complexes, analyzed by 4% polyacrylamide gel, depicted in Fig. 3, were quantified by computer scanning of the autoradiography. The numbers represent the percentage of filter retained GRE–protein complexes in the absence, or the presence, of 100 and 150 ng of calf thymus DNA (ctDNA).

not show competition [Fig. 7(A) and (B, lane 4)]. If the mitochondrial extract is incubated with monospecific EP anti-GR polyclonal antibody before carrying out the binding reaction with GREa [Fig. 7(A, lane 5)] a gel shift can be detected, demonstrating that one of the proteins in the mitochondrial extract which bind to GREa, is GR.

The results presented in Figs 6 and 7 show that: (i) purified GR from rat liver can bind to both GREs from the D-loop area of the mitochondrial genome; and (ii) the mitochondrial extract from liver of dexamethasone-induced mice contain protein(s) which specifically bind to both GREs. One of these proteins is GR (same gel retardation band as with purified GR and gel shift in the presence of monospecific EP polyclonal antibody).

DISCUSSION

Mitochondrial biogenesis and metabolism require the coordinated expression of numerous genes occurring in two different cell compartments—the nucleus and the mitochondria. Sharing of the same *cis* elements and *trans*-acting factors by the mitochondrial and the nuclear transcriptional regulatory apparatus is a possible way of coordinating transcription of the physically separated genes.

The influence of steroid hormones, especially glucocorticoids and thyroid hormones, both on nuclear and mitochondrial metabolism, is well documented and the question arises concerning coordination of nuclear and mitochondrial events by these regulatory agents. Previous reports have shown a rapid entrance of cortisol in rat liver mitochondria, mostly in unmetabolized form [1, 14], as is the case with the import of the hormone in the nucleus; and a rapid stimulation of mitochondrial RNA synthesis wtih similar kinetics, as is the stimulation of nuclear transcription [1, 15–17]. Furthermore, sequences showing strong similarity to GREs, but also to estrogen and thyroid hormone response elements [30], have been detected in the mitochondrial genome. The above findings have led us to propose that steroid and thyroid hormones, well established important nuclear transcription regulatory factors, can, parallel to their action on the nuclear genome, also exert a primary action on mitochondrial genes [30]. Glucocorticoids exert their action on the nuclear genome by binding to

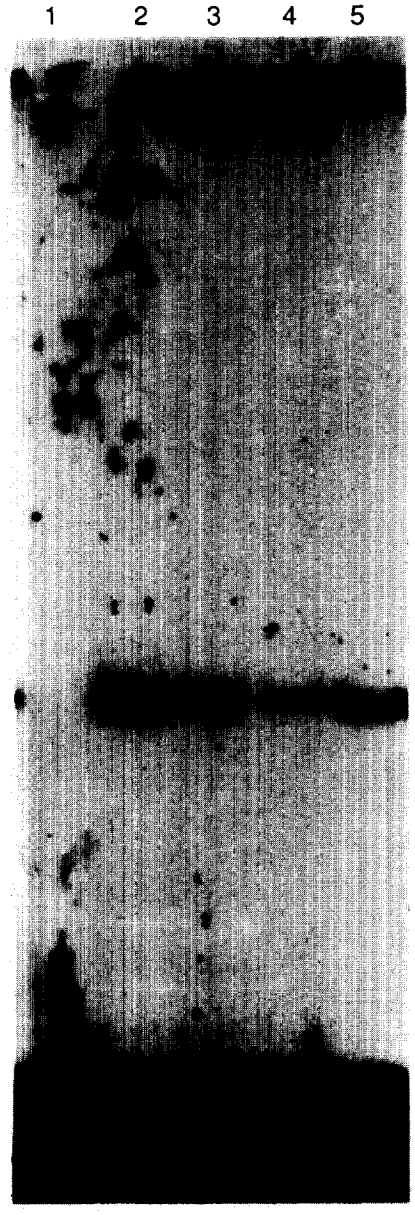


Fig. 4. Interaction of purified GR from rat liver cytosol with the GRE from MMTV-LTR. 1 ng of ^{32}P end-labelled 57 bp fragment containing GRE from MMTV-LTR (50,000 cpm) was incubated with purified rat liver GR (20 ng), either in the presence of specific competitor (57 bp non-labelled fragment of MMTV-LTR; 25 ng, lane 3 and 50 ng, lane 4), non-specific competitor (126 bp fragment from the 21H plasmid; 50 ng, lane 5) or without any competitor (lane 2). Lane 1 depicts the ^{32}P end-labelled 57 bp fragment alone. After the DNA binding reaction, the samples were analyzed on 4% non-denaturing polyacrylamide gel. The DNA binding and gel retardation assay were performed as described in Materials and Methods, electrophoresis and autoradiography as described in Fig. 2.

the cytoplasmic receptor protein (GR) forming a GR complex which, after dimerization, binds to the GREs and modulates gene expression [20, 43]. We postulated that the same, or a similar mechanism, could apply in the case of glucocorticoid, of other steroids and of thyroid hormone action on the mitochondrial genome. We have recently demonstrated *in vivo* and *in vitro*, that after dexamethasone induction, the rat liver GR, initially localized in the cytoplasm, rapidly translocates not only to the nucleus, but also to the mitochondria, with similar kinetics [32]. It is reasonable to expect that the GR, after entering the mitochondria, will bind to the GREs, as it does in the nucleus.

The data presented in this paper provide evidence for this model. We investigated GREs present in COX genes and in the D-loop. We first tested the ability of putative mitochondrial GREs I–IV present in the COX subunit I and III genes, and GREa and GREb, present in the D-loop region, to function as specific binding sites for GR from rat liver cytosol by the filter retention method or by gel shift assays. We focused our interest on these six putative GREs having in mind that dexamethasone treatment increases the total amount of mitochondrial RNA and specifically the mRNA for COX subunit I and II [1, 15–17]. Using both methods we have shown that GR specifically interacts with all six potential mitochondrial GREs (Figs 2, 5 and 6). This is supported by the results presented in Fig. 4 showing the same band shift of purified GR with GRE from MMTV-LTR.

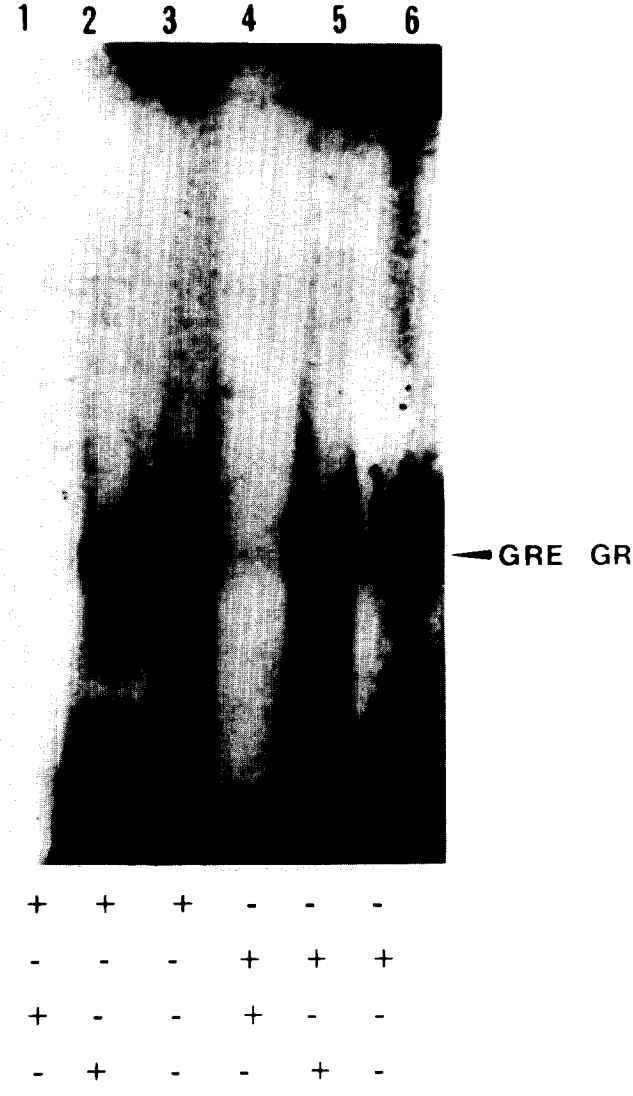


Fig. 5. Interaction of purified GR from rat liver cytosol with mitochondrial putative GREs (GREI and GREIII). Mitochondrial ^{32}P end-labelled fragments, GREI (40 bp, 1 ng, 15,000 cpm) and GREIII (34 bp, 1 ng, 15,000 cpm) were used as probes in the gel retardation assay with purified rat liver GR (40 ng). Samples were analyzed on 5% non-denaturing polyacrylamide gel electrophoresis. GREI and GREIII were incubated with purified GR either in the presence of specific competitor (57 bp fragment of MMTV-LTR, 7 ng, lanes 1 and 4), non-specific competitor (126 bp fragment from the 21H plasmid, 18 ng, lanes 2 and 5) or without any competitor (lanes 3 and 6). The band resulting from GR binding to the GREs is indicated by GRE–GR. The DNA binding reaction and gel retardation assay were performed as described in Figs 2–4 and in Materials and Methods.

We further investigated the binding and the specificity of binding of proteins present in liver mitochondrial extracts of dexamethasone-induced mice to all six putative GREs. Using the same methodology we were able to show that liver mitochondrial extracts of dexamethasone-induced animals contain protein(s) which bind to all six putative GREs. Furthermore, we demonstrated by competition experiments that the binding is specific. We also showed by super gel shift, incubating the mitochondrial extracts with monospecific EP polyclonal antibody before binding with the DNA probe, that the protein in the mitochondrial extract which binds to the GREs is GR. The gel retardation assay also leads to the conclusion that the mitochondrial protein which binds to the GREs is GR, since the same gel retardation band was obtained using either GR or mitochondrial extract.

Results in gel retardation assays, similar to those presented here, have been obtained in the study of the interaction of nuclear and mitochondrial extracts from two cell lines, MCF7 and LATK$^-$, with two synthetic oligonucleotides, corresponding to GRE I and II of the mitochondrial COX subunit I gene, and to an oligonucleotide, containing the sequence of the human metallothionein IIA GRE [44].

The above findings, that all six putative mitochondrial GREs are specific binding sites for GR, raise questions about the role of these potential GREs in the regulation of mitochondrial gene expression. It is known that the circular mtDNA shows differences, both in structure and expression, compared to the nuclear genome. The mitochondrial genome does not have introns and the genes are transcribed in a way reminiscent of bacterial operons. Two out of the six GREs investigated in this paper (GREa and b) reside in the D-loop area, which contains the mitochondrial promoters. A logical assumption is that GREs a and b are involved in the regulation of mitochondrial transcription and that the same transcription factor (GR) can regulate in a similar way nuclear and mitochondrial

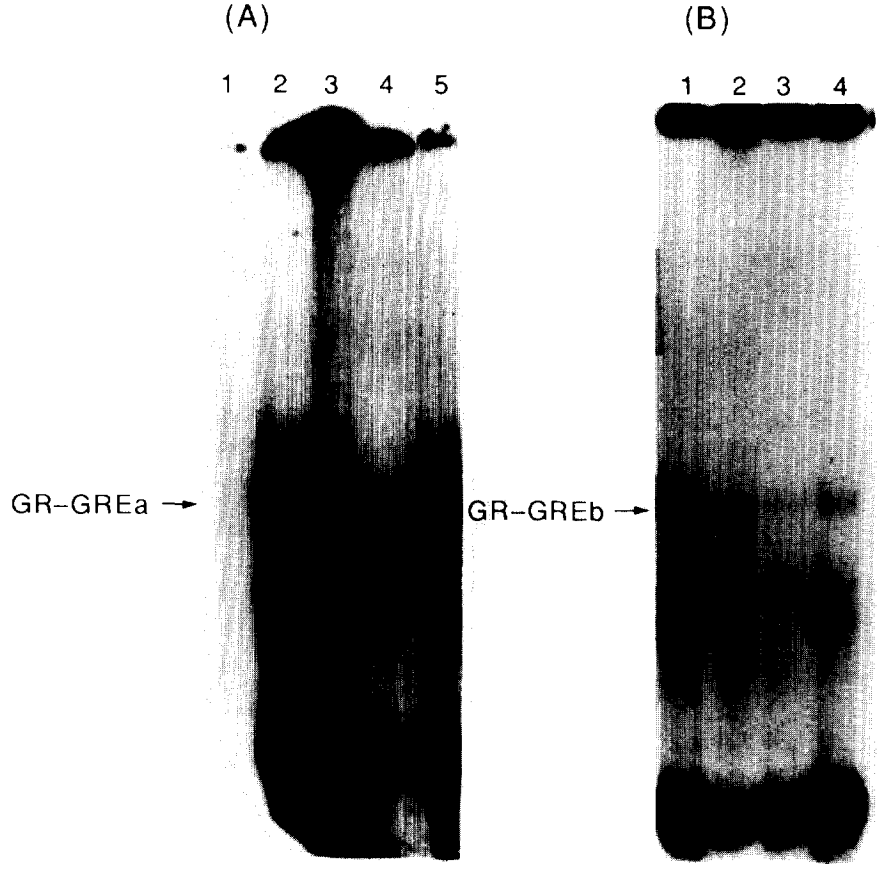


Fig. 6. Gel retardation analysis of the binding of purified GR from rat liver to the synthetic GREa and GREb oligonucleotides. (A) Binding of purified GR (20 ng) to GREa (3 ng, 40,000 cpm) (lanes 2–5), in the absence of competitor, lane 2; in the presence of competitor 150 bp fragment (originated from 2 × GRE37TK plasmid, 15 ng), lane 3; in the presence of the 400 bp fragment (originated from 2 × GRE37TK plasmid, 10 ng), lane 4; and in the presence of the p59 bp synthetic oligonucleotide (18 ng), lane 5. Lane 1 represents GREa in the absence of protein. (B) Binding of purified GR (20 ng) (lanes 1–4) to the GREb (3 ng, 40,000 cpm), in the absence, lane 1, or presence of competitors: 150 bp fragment (15 ng), lane 2; 400 bp fragment (10 ng), lane 3; and p59 bp synthetic oligonucleotide (18 ng), lane 4. Following the incubation of the putative GREs with purified GR, the samples were loaded on a 5% polyacrylamide gel. The DNA binding reaction, electrophoresis and autoradiography were performed as described in Figs 2–5. The band resulting from GR binding to the oligonucleotides is indicated as GR–GREa(b).

gene transcription. The findings of Suzuki *et al.* [45] are worth mentioning, who showed the presence of similar elements in the transcriptional regulatory regions of human nuclear and mitochondrial genes involved in the oxidative phosphorylation system. These authors proved that two out of three nuclear regulatory elements (Mt3 and Mt4), located at the 5′-flanking regions of the human nuclear genes for subunits of the mitochondrial cytochrome bc1 complex, are found also in the D-loop of the mammalian mitochondrial genome. It was ascertained that the nuclear protein factors which recognize the Mt3 and Mt4 elements in the nuclear gene, specifically bind to the respective mitochondrial genome elements. Their results provide the evidence that the Mt3 and Mt4 *cis*-elements and the respective *trans*-acting protein regulatory factors are shared by the two genetic systems, and that the *trans*-acting factors are involved in communication between the nuclear and mitochondrial genomes. Our results strongly suggest a similar role of glucocorticoids on the nuclear and mitochondrial genomes as previously postulated [30–32].

Intriguing is the existence and position of the other four putative GREs which are located within the mitochondrial genes coding for the COX subunits I and III. The question of their functional role is raised. Do these GREs play a role in the modulation of transcription, or could they be involved in the elongation or stability of nascent transcripts? These questions are under consideration in our laboratory and are being explored using, among others, chloramphenicol acetyltransferase (CAT) transfection studies, in which competent cells are transfected with constructs composed of the CAT gene linked to the putative mitochondrial GREs. We have already obtained positive results using constructs of GREa and GREb.

The results presented in this paper and previous papers [30–32 and 44], together with a large body of evidence on the effects of glucocorticoids on mitochondrial metabolism and mitochondrial RNA and protein

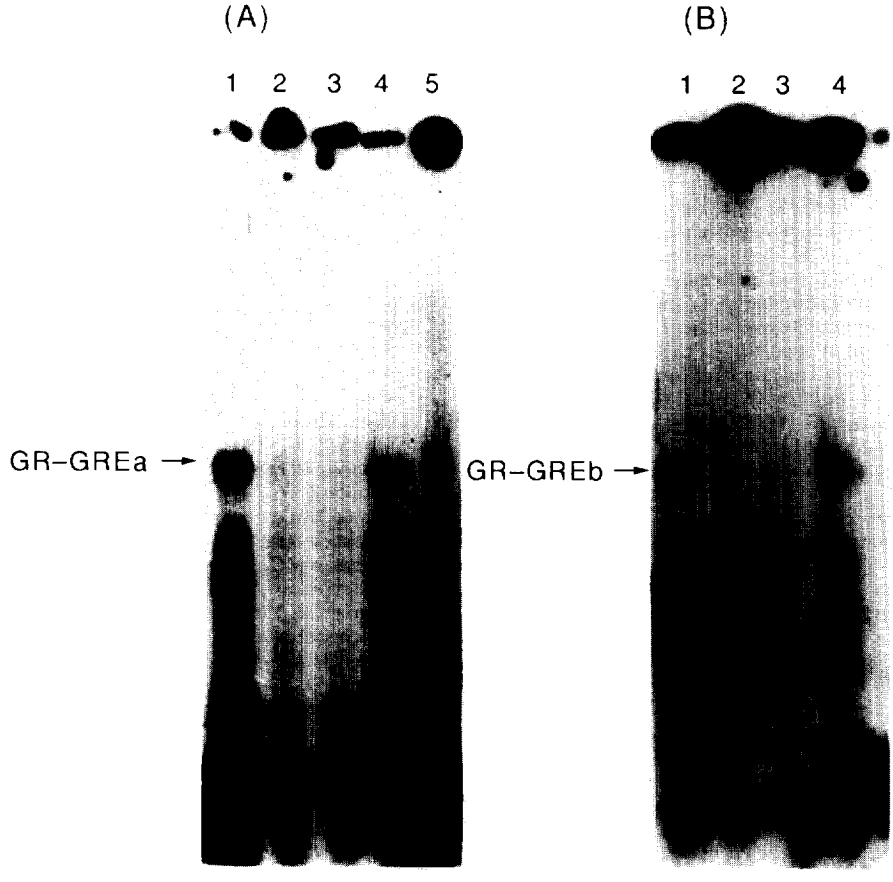


Fig. 7. Gel retardation analysis of the binding of protein(s) from mitochondrial extract to GREa and GREb. (A) Binding of protein(s) from the mitochondrial extract (5 μg) to GREa (3 ng, 40,000 cpm), in the presence (lane 1), or presence of competitors: 150 bp fragment (50 ng, lane 2), 400 bp fragment (10 ng, lane 3), and p59 bp synthetic oligonucleotide (18 ng, lane 4). In lane 5, the mitochondrial extract was first incubated with monospecific EP polyclonal antibody and then with GREa. (B) Binding of protein(s) from the mitochondrial extract (5 μg) to GREb (3 ng, 40,000 cpm), in the absence (lane 1), or presence of competitors, 150 bp fragment (50 ng, lane 2), 400 bp fragment (10 ng, lane 3), and p59 bp synthetic oligonucleotide (18 ng, lane 4). Following the incubation of GREs with the mitochondrial extract, the samples were loaded on a 5% polyacrylamide gel. The DNA binding reaction, electrophoresis and autoradiography were performed as described in previous figures. The band resulting from protein(s) binding to the oligonucleotides is indicated as GR–GREa(b).

synthesis, favour our hypothesis that glucocorticoids, and, probably other ligands, acting through the superfamily of steroid receptors directly, and not necessarily through secondary nuclear signals, regulate mitochondrial genome activity. This could represent one general mechanism of a direct action of common nuclear transcription factors, independently, but in a coordinated way, on both the nuclear and the mitochondrial genome [45].

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