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Life Sciences xx (2005) xxx – xxx
Life Sciences
www.elsevier.com/locate/lifescie

Thyroid hormone receptor β_1 gene expression is increased by Dexamethasone at transcriptional level in rat liver☆

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Received 9 May 2005; accepted 11 October 2005

Abstract

Triiodothyronine (T_3) exerts most of its effect through nuclear thyroid hormone receptors (TR) which bind mainly as heterodimers with retinoid-X receptors (RXR) to thyroid hormone response elements (TRE) in target genes. It is well known that the synergistic interaction of T_3 and glucocorticoids has a role on the synthesis of growth hormone in rat pituitary cell lines and in the T_3-induced metamorphosis in amphibians. Glucocorticoids increased mRNAs of T_3-regulated hepatic genes. Our laboratory reported increased specific metabolic actions of T_3 in rat liver by Dexamethasone (Dex) through a mechanism involving an up-regulation of the maximal binding capacity of TR.

In this study we further explored the participation of TR in the molecular mechanism of the Dex-induced increase on liver T_3-specific metabolic action. Dex administration to adrenalectomized rats induced an increase of liver TRβ_1 protein and mRNA. Nuclear run-on assay revealed that Dex up-regulated the TR gene transcriptional rate. Transfection assay in COS-7 cells indicated that Dex increased the transcriptional activity of the TRβ_1 promoter. Electrophoretic mobility shift assay demonstrated that Dex induced the binding of additional proteins related to or neighboring the DNA sequence of a glucocorticoid receptor (GR) binding (GRE) half-site in the TRβ_1 promoter. Evidences for an interaction of GR on the TRβ_1 promoter have been obtained. Moreover, the specificity of the GR binding to GRE was determined not only by the GRE DNA sequence, but also by the interaction of the GR with other transacting factors bound to sequences flanking the GRE.

Keywords: Thyroid hormone receptor; TR expression; Dexamethasone; Glucocorticoid receptor

Introduction

Thyroid hormones are essential for normal growth, development, differentiation and metabolism. These effects are mediated mainly by thyroid hormone receptors (TR), which activate or repress the transcription of specific target genes in a ligand-dependent manner. TR belong to the nuclear receptor superfamily which include steroid, retinoid acid and vitamin D_3 receptors. TR are encoded by two different gene loci, *TRA* and *TRB*. The *TRA* gene encodes for TRα_1, the non-hormone binding splicing variant TRα_2 and the truncated products TR$\Delta\alpha_1$ and TR$\Delta\alpha_2$. Through alternative promoter usage, the *TRB* gene yields TRβ_1, TRβ_2, TRβ_3 and the truncated variant TR$\Delta\beta_3$ which are unable to bind the thyroid hormone (Bassett et al., 2003). TRα_1 and TRβ_1 are ubiquitously expressed. TR bind mainly as heterodimers with retinoid-X-receptors (RXR) to thyroid hormone response elements (TRE) in the promoter region of thyroid hormone-target genes. Several lines of evidence suggest that RXR are the major TR partners and thus may play a critical role in triiodothyronine (T_3) mediated transcription (Yen, 2001; Ikeda et al., 1996).

One of the major target tissues for thyroid hormone action is the liver, where genes from different metabolic pathways such as Spot 14 (Zilz et al., 1990), malic enzyme (ME) (Petty et al., 1990) and type I 5′deiodinase (5′DI) (Toyoda et al., 1995) are

☆ Part of these studies was presented at the X Latin American Thyroid Congress, Villa Carlos Paz, Córdoba, Argentina, 2003.

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0024-3205/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2005.10.019

regulated by T_3 at the transcriptional level. This action has been demonstrated to be mediated by TRβ_1, the major functional TR in liver, accounting for 80% of hepatic T_3-binding activity (Weiss et al., 1998).

A characteristic of T_3 action is the multihormonal interaction in the final expression of a specific metabolic effect. Several reports indicated that T_3 increases rat growth hormone (GH) synthesis and that glucocorticoids synergistically stimulate T_3 action on the GH expression in rat pituitary cell lines (Liu et al., 2003). Diverse reports have also demonstrated that glucocorticoids increase the mRNAs of T_3-regulated hepatic genes such as Spot 14, ME and 5′DI (Yamaguchi et al., 1999; Molero et al., 1993; Menjo et al., 1993). This enhanced T_3 action by glucocorticoids was abolished by pretreatment with cycloheximide, suggesting that glucocorticoids induce some protein factor(s) that contributes to the enhancement of the transcriptional action of T_3. Dexamethasone (Dex), a synthetic glucocorticoid, has been reported to potentiate the T_3-induced metamorphosis and increase TR and RXR mRNA and protein levels in *Xenopus* tadpole tails (Iwamuro and Tata, 1995).

Previous in vivo studies carried out in our laboratory demonstrated that Dex resulted in a time- and dose-dependent increase in the activity of ME, a specific metabolic marker of T_3 action in rat liver. Under these circumstances, a simultaneous increase in the maximal binding capacity of liver TR was observed (Recúpero et al., 1983, 1986).

Since the interaction between T_3 and its receptors in the cell nucleus is the first step in the chain of molecular events leading to a specific response to the hormone, TR play a crucial role in the mechanism of thyroid hormone action (Tata, 2000). The goal of this study was to explore the participation of TR in the molecular mechanism of the Dex-induced increase of liver T_3-specific metabolic action in rat liver. We evaluated the impact of Dex administration on liver TRβ_1 protein level, TRβ_1 mRNA expression and the transcriptional rate of TR gene. We also explored the effect of Dex on TRβ_1 promoter activity and protein/DNA interaction.

Materials and methods

Materials

Dexamethasone (Dex) and 3, 3′,5 Triiodo-L-thyronine (T_3) were from Sigma Chemical Co., St. Louis, MO, USA. [α^{32}P]dATP and Renaissance Enhanced Luminol Western Blot Chemiluminescence reagent from Du Pont NEN, Boston, MA, USA; nitrocellulose (NC) membranes (Trans-Blot Transfer Medium, 0.45 μm pore size) from Bio-Rad Laboratories, Hercules, CA, USA. The DNA labeling kit (Preme-a-gene Labeling System) from Promega Corp, WI, USA. Radiographic films from Eastman Kodak, Rochester, NY, USA and intensifying screens from Amersham International plc, Buckinghamshire, UK. Anti-TR, anti-actin, anti-GR and anti-RXRα antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The plasmid pBSK$^+$ TRβ_1 was generously donated by Dr. R. Koenig, Department of Internal Medicine, Endocrinology and Metabolism Division, The University of Michigan Medical Center, MI, USA. The plasmid pGL-2 TRβ_1 promoter/luciferase was generously donated by Dr. L.J. De Groot, Department of Medicine, Thyroid Study Unit, The University of Chicago, Chicago, USA and TRβ_1 and GR expression vectors by Dr. A. Aranda, Consejo Superior de Investigaciones Científicas, Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, España. All chemicals were of reagent grade.

Animals and Dexamethasone (Dex) treatment

Adult male Wistar rats (250±50 g) had free access to a balanced diet and tap water, and were maintained under a 12 h light cycle. Animals were adrenalectomized surgically and maintained on 1.0% NaCl in the drinking water for 7 days. Rats were injected i.p. with vehicle (0.15 M NaCl, control group) or Dex every 12 h for 48 h. At the end of the treatment, rats were fasted for 12 h, killed by decapitation, and liver removed and processed for different purposes. Animal protocols were in compliance with the Guidelines of NIH and the local Institutional Animal Care Committee.

Isolation of the nuclear fraction and preparation of the nuclear extract

Nuclear extracts were prepared according to Sugawara et al. (1993). Briefly, 1 g of liver was placed in 10 ml 0.32 M sucrose, 3 mM $MgCl_2$, 2 mM EDTA, 10 mM dithiothreitol (DTT) and a protease inhibitor cocktail (0.1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/ml Pepstatin A, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, 40 μg/ml Spermine, 0.26 mg/ml Spermidine) at 4 °C and homogenized with a teflon-glass motor-driven tissue grinder. The homogenate was centrifuged at 1000 ×*g* for 10 min. The pellet was resuspended in the same volume of 2.3 M sucrose, 1.5 mM $MgCl_2$, 10 mM DTT and the protease inhibitor cocktail and layered over a 1 ml cushion of the same buffer in polyallomer tubes. The suspension was centrifuged in a Beckman SW-41 rotor at 4 °C at 37,000 rpm (Beckman Instruments, Palo Alto, CA, USA) for 1 h. The pellet obtained was suspended in 10 ml 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5), 1 mM DTT and the protease inhibitor cocktail (SMCT buffer). Then, 500 μl of 10% (vol/vol) Nonidet P-40 were added, and the solution was incubated on ice for 10 min and centrifuged at 250 ×*g* for 5 min. The pellet was washed once with SMCT before resuspending in 2 vol 20 mM HEPES (pH 7.8), 0.6 M KCl, 0.02 mM $ZnCl_2$, 0.2 mM EGTA, 0.5 mM DTT, and the protease inhibitor cocktail and incubated on ice for 30 min. The suspension was centrifuged at 20,000 ×*g* for 30 min at 4 °C, and the supernatant, containing the high salt nuclei extract, was dialyzed against 20 mM HEPES (pH 7.8), 5 mM 2-mercaptoethanol, 50 mM NaCl, 2 mM EGTA, 10% (vol/vol) glycerol, and 0.1 mM PMSF overnight and centrifuged at 10,000 ×*g* for 15 min. Aliquots were stored at −80 °C until use for different purposes. Total protein concentration was measured by the Bradford technique (Bradford, 1976).

Western blot

40 μg proteins from nuclear extracts were electrophorezed through a 4% stacking, 8% resolving SDS-polyacrylamide gel, electroblotted in 48 mM Tris–HCl (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol on nitrocellulose (NC) membranes. Membranes were blocked for non-specific binding with 5% non-fat milk–PBS–0.1% Tween 20, then washed briefly in PBS and incubated with anti-TR (1:500 dilution) or anti-actin (1:1000 dilution). The anti-TR (FL-408, Santa Cruz Biotechnology sc-772) was a rabbit polyclonal antibody that reacts with chicken, mouse, rat and human TRα1 (47 KDa) and $TR\beta_1$ (55 KDa). The anti-actin (I-19, Santa Cruz Biotechnology sc-1616) was a goat polyclonal antibody that reacts with mouse, rat, human, *Zebrafish, C. elegans, Drosophila, S. cerevisiae* and *Xenopus* Actin (43 KDa). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit: Santa Cruz sc-2004, 1:15,000 dilution and rabbit anti-goat sc-2768, 1:10,000), and bands were revealed by the enhanced chemiluminescence protocol (Du Pont NEN NEL-100). Bands intensities were determined by scanning densitometry (Scion Image Software, Scion Corporation, NIH, Baltimore, USA). Molecular weight (MW) markers were run in parallel lanes. Control for protein loading was revealed by Western blot analysis of the nuclear extracts using anti-actin antibody.

Total RNA extraction

Total RNA was prepared by the one-step acid-guanidinium method of Chomczynski and Sacchi (1987). In brief, 1 g of liver was homogenized in 10 ml denaturing solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% *N*-lauroyl sarcosine and 0.1% β-mercaptoethanol). After phenol–chloroform–isoamyl alcohol (50:49:1) extraction, RNA was precipitated in isopropanol, recovered by centrifugation and washed; RNA was dissolved in diethyl pyrocarbonate-treated water, quantified and checked for purity by spectrophotometry at 260 and 280 nm.

Reverse transcription (RT)-polymerase chain reaction (PCR)

Messenger RNA was reversed transcribed and amplified by PCR (RT-PCR) according to standard methods (Ausubel et al., 1996b) with some modifications. Briefly, 1 μg of total RNA was incubated with 0.1 μM of degenerated oligo dT12VG primer at 65 °C for 3 min. After 3 min on ice, the following reagents were added: 20 U RNase inhibitor (RNaseOUT, Promega, Madison, WI), 4 μl of 5× RT buffer (250 mM Tris–HCl, pH: 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 0.5 mM of each dNTP and 200 U M-MLV RT (Moloney murine leukemia virus reverse transcriptase, Promega, Madison, WI). After 1 h at 37 °C, remnant M-MLV RT was inactivated at 95 °C for 5 min. The expression of TRβ1 mRNA was normalized with the housekeeping β-actin mRNA that was measured in parallel tubes. For PCR, primer design and optimizations were carried out with software downloaded from the National Center for Biotechnology Information and Jellyfish, 3.0 version. GenBank accession no. NM 009380 was used for designing rat TRβ1 primers and NM 031144 was used for β-actin primers. Primers were designed to distinguish cDNA and genomic DNA/pseudogenes (Kreuzer et al., 1999). Primers were from Sigma (Sigma-Aldrich from Buenos Aires, Argentina) and designed to amplify a 370 bp for TRβ1 mRNA and a 273 bp for β-actin mRNA; according to the following sequence:

Target	Denomination	Primer sequence	Nucleotide
$TR\beta_1$	$TR\beta_1$-S	5′-CAGTGCCAGGAATGTCGCTTTAAG-3′	822
	$TR\beta_1$-AS	5′-ACTCTGGTAATTGCTGGTGTGATGAT-3′	1192
β-actin	β-actin-S	5′-CGGAACCGCTCATTGCC-3′	832
	β-actin-AS	5′-ACCCACACTGTGCCCATCTA-3′	559

PCR was carried out in a 20 μl final vol: 1.5 mM $MgCl_2$, 4 μl 5× PCR buffer, 1 U Taq-polymerase (Promega, Madison, Wisconsin), 0.25 mM each dNTP (Promega, Madison, Wisconsin) and 2 μl RT product. TRβ1 cDNA was amplified with 0.5 μM sense and antisense TRβ1 primers, and β-actin cDNA with 0.25 μM primers. A negative control (sterile water instead of RT product) was included in each PCR run. The PCR amplification was performed on a ICycler PCR System (Bio-Rad, Hercules, California). The thermal profile was: 94 °C 5 min; (34× for $TR\beta_1$ and 26× for β-actin): 94 °C 1 min, 56 °C 1 min; 72 °C 2 min; and 72 °C 10 min. The mass of total RNA for RT, the number of cycles for PCR and $MgCl_2$, primer and dNTP concentrations were selected experimentally (data not shown). RT-PCR products were resolved by electrophoresis in 2% agarose gel followed by ethidium bromide staining. Band intensities were determined by scanning densitometry (Scion Image Software, Scion Corporation, NIH, Baltimore, USA) and $TR\beta_1$ mRNA level was expressed as the densitometry of each TRβ1 signal normalized to that of the β-actin in the same sample.

Nuclear run-on assay

Nuclear run-on assay was performed as described by Groudine et al. (1981) with slight modifications (Greenberg and Bender, 1997). Nuclei from liver tissue were isolated as described above using RNase free solutions and frozen at −80 °C in glycerol storage buffer (50 mM Tris–HCl, pH 8.3; 40% (v/v) glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA) until assayed. The reaction volume (200 μl) contained 5×10^7 nuclei, 30% glycerol, 2.5 mM DTT, 1 mM $MgCl_2$, 70 mM KCl, 0.25 mM guanosine triphosphate and cytosine triphosphate, 0.5 mM adenosine triphosphate, and 200 μCi of uridine [α^{32}]-triphosphate (450 Ci/mmol, New England Nuclear). Nuclei were incubated for 30 min at 30 °C with shaking, and the reaction terminated by the addition of 20 μg/ml deoxyribonuclease I (DNase I)–ribonuclease (RNase) free (Promega) and incubated at 30 °C for 5 min. The reaction was deproteinized by incubation at 42 °C for 30 min in 1% SDS, 5 mM EDTA and 10 mM Tris–HCl pH (7.4) with 100 μg/ml proteinase K solution and subsequent phenol–chloroform extraction. Yeast

transfer RNA was added to the resultant aqueous phase to a final concentration of 100 μg/ml and the aqueous phase precipitated at 4 °C with 5% cold trichloroacetic acid (TCA) in the presence of 30 mM $Na_2P_2O_7$. After 30 min, the precipitate was collected by suction on 25 mm (0.45 μm pore size) NC filter disks (Millipore) and washed three times with 10 ml of 3% TCA and 30 mM $Na_2P_2O_7$. Disks were transferred to glass scintillation vials, and 0.9 ml of DNase buffer (20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$) and 25 μg of DNase I–RNase free added and the reaction incubated for 30 min at 37 °C. DNase digestion was terminated by the addition of 15 mM EDTA and 1% SDS. RNA was then eluted from the disks by incubating the filter in the above solution at 65 °C for 10 min. After removal of the solution, the filter was incubated in 0.5 ml of 1% SDS–10 mM Tris–HCl (pH 7.5)–5 mM EDTA for an additional 10 min. The two solutions were combined, incubated at 37 °C in the presence of 25 μg/ml proteinase K for 30 min, and extracted twice with equal volumes of phenol–chloroform and twice with chloroform. The final aqueous phase was precipitated at −20 °C with 0.1 M NaCl and 2.5 volumes of ethanol. The precipitate was recovered by 30 min centrifugation at 11,000 rpm in the HB-4 rotor of a Sorval RC-5 centrifuge and suspended in 50 μl of 10 mM Tris–HCl (pH 7.5) and 2 mM EDTA.

Transcripts containing TR sequences were quantified by hybridization to NC membranes containing immobilized TR cDNA ($pBSK^+$ $TR\beta_1$ linearized with *Eco*RI), $pBSK^+$ plasmid vector (linearized with *Hin*dIII) and pBR 322 18 S rRNA (linearized with *Hin*dIII) (2 μg each) previously immobilized using a dot blot apparatus (Hybri Dot 96 Well Filtration Manifold, Gibco BRL, Gaithersburg MD, USA). The NC paper, cut into strips, was placed in a polyethylene bag with 1 ml prehybridization buffer consisting of 50% deionized formamide, 5× standard saline citrate (SSC), 0.1% SDS, 5× Denhardt's solution and denatured salmon sperm DNA (200 μg/ml) for 4 h at 42 °C. Hybridization with [α^{32} P]-RNA was performed at 42 °C in prehybridization buffer and 50% (wt/vol) dextran sulfate (4:1). The RNA was denatured at 65 °C for 5 min, cooled on ice, and incorporated into hybridization buffer before its addition to the blot. Hybridizations were conducted for 4 days at 42 °C, blots washed with four changes of 2× SSC–0.1% SDS for 5 min each at room temperature and washed twice with 0.3× SSC–0.1% SDS for 15 min at 65 °C. The strips were exposed to Kodak X-Omat film at −80 °C with intensifying screens for 4 days. Bands intensities were determined by scanning densitometry (Scion Image Software, Scion Corporation, NIH, Baltimore, USA) and the level of each TR mRNA expressed as the absorbance of the TR signal minus that of $pBSK^+$ vector normalized to the 18S rRNA in the same sample.

Cell culture, transfection and luciferase assay

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen/Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, antibiotics and glutamine at 37 °C in a humidified incubator (5% CO_2). Cells were seeded at 3×10^5 cells in a 60 mm dish 24 h prior to transfection. Cells were transfected with 5 μg of reporter plasmid (pGL-2-$TR\beta_1$ promoter: −1325 to +44/luciferase), 1 μg of pCDM8-$TR\beta_1$ expression vector and 1 μg of pRSV-GRα expression vector by calcium phosphate coprecipitation as previously described (Ausubel et al., 1996a). 1 μg of pRSV-β-galactosidase was added to all transfections to monitor the efficiency of DNA uptake by the cells. The promoterless pGL2-basic was transfected as a negative control. After 2 h of incubation with the precipitate, cells were shocked with 15% glycerol in phosphate-buffered saline, and then maintained in DMEM medium supplemented with 10% fetal bovine serum stripped of T_3 by constant mixing with 5% (w/v) Dowex 1× 8-400 resin (Sigma, St. Louis, USA) overnight before ultrafiltration. Twenty four hours after transfection, cells were treated with T_3 and Dex. Transfected cells were lysed and assayed for reporter gene activity using luciferase assay reagent according to manufacturer's instruction (Promega, Madison, WI, USA). Luciferase activity was measured in a TD 20–20 luminometer (Turner Designs, Sunnyvale, CA, USA). β-galactosidase activity was measured with *o*-nitrophenyl β-D-galactopyranoside as substrate.

Over-expression of GR in COS-7 cells was performed by transient transfection using 5 μg of pRSV-GRα expression vector. Twenty four hours after transfection, cells were harvested and lysed in buffer containing 20 mM Tris–HCl pH 7.8, 20% glycerol, 400 mM KCl, 2 mM DTT and protease inhibitors to obtain protein extracts for electromobility shift assays.

Electromobility shift assay (EMSA)

Nuclear extracts were prepared as described above. Synthesized double-stranded oligonucleotides: −850/−824 fragment of 5′-flanking region of the $TR\beta_1$ promoter ($TR\beta_1$ −850/−824): 5′ GAGGCCCCA AGAAGA GCCAGAGCGCC 3′ (consensus sequence for GR binding *site 1* is underlined) and −678/−652 fragment of 5′-flanking region of the $TR\beta_1$ promoter ($TR\beta_1$ −678/−652): 5′ CATTCTTTCC TCTTCT CCAACTAGAG 3′ (consensus sequence for GR binding *site 2* is underlined) were end labeled with [α-^{32}P] dATP by using the Klenow fragment of *E. Coli* DNA polymerase and purified in a Sephadex G25 (Sigma, St. Louis, USA) column. 5 μg of nuclear extracts from rat liver or 15 μg of COS-7 whole protein extracts were incubated in a 20 μl reaction volume for 15 min on ice in binding mix buffer containing 40 mM HEPES pH 7.9, 200 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 1 μg poly (dI-dC) and 5% ficoll. Radiolabeled probe (50,000; ~50 pg DNA) was added, and incubation continued for 30 min at room temperature. DNA–protein complexes were resolved on 5% native polyacrylamide gels containing 0.5% Tris borate–EDTA buffer (TBE 10%: 890 mM Tris, 890 mM boric acid, 20 mM EDTA). The gels were dried and autoradiographed at −80 °C. For competition experiments, the protein fractions were preincubated with the appropriate unlabeled oligonucleotide (100×) before addition of the labeled probe. Double-stranded

oligonucleotides used for competition were as follow: the oligonucleotide TRβ$_1$ −850/−824 containing the mutated GRE: Mut A: 5′ GGAGGCCCCA **ATGGCG** GCCA-GAGCGC 3′ (mutated sequences are in bold); the oligonucleotide TRβ$_1$ −850/−824 containing the mutated GRE and flanking sequences: Mut B: 5′ GGAGGC**GATAATGGCG**G-C**AG**GAGCGC 3′ (mutated sequences are in bold); an Consensus GRE: 5′ **AGAACA**GAG**TGTTCT** 3′ (nucleotides in bold corresponds to the consensus motifs for GRE). Where indicated, nuclear extracts were incubated with antibodies for 1 h on ice before adding the radiolabeled probe and processed as indicated above. The anti-GR (M-20, Santa Cruz Biotechnology sc-1004) was a rabbit polyclonal antibody that reacts with mouse and rat GR. The anti-RXRα (D-20, Santa Cruz Biotechnology sc-553) was a rabbit polyclonal antibody that reacts specifically with RXRα.

Statistical analysis

Comparisons between two groups were made using the Student's *t* test. Analysis of intergroup differences was conducted by one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test. A $p<0.05$ was considered statistically significant.

Results

Dex increased rat liver TRβ$_1$ protein

As depicted in Fig. 1A liver TRβ$_1$ protein level after Dex administration was visualized as a doublet of 52 and 55 KDa, a finding in consonance with previous reports in liver from rats and human beings (Tagami et al., 1993; Chamba et al., 1996). No signal for TRα was found in accordance with the scarce TRα abundance previously described in liver (Tagami et al., 1993). The densitometric analysis of the corresponding bands (Fig. 1B) revealed that TRβ$_1$ protein level was significantly increased by 250 μg Dex/100 g BW for 48 h (52% over control values). In contrast, no significant difference in TRβ$_1$ level was attained after Dex treatment with lower doses (50 and 125 μg/100 g BW). Actin signal was used as control of protein loading (Fig. 1A, lower panel).

Normal sham-operated animals were not included in these studies since previous results carried out in our laboratory demonstrated no significant difference in the maximal binding capacity of TR between sham-operated and adrenalectomized rats (Recúpero et al., 1983).

Dex augmented rat liver TRβ$_1$ mRNA

Fig. 2A shows a representative RT-PCR assay of total liver RNA from control and Dex-treated rats, revealing an amplified band of approximately 400 bp consistent with the expected size for TRβ$_1$ transcript amplified with the designed primers (370 bp). The densitometric analysis (Fig. 2B) revealed a significant increase in TRβ$_1$ mRNA after the administration of 125 and 250 μg/100 g BW Dex in a dose-dependent manner (43% and

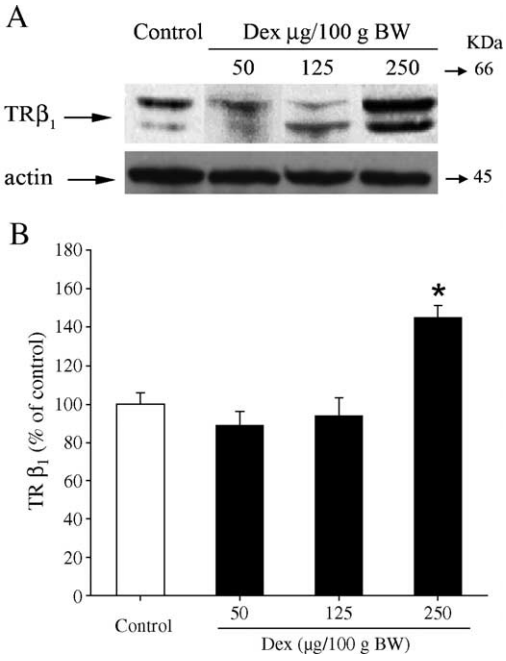

Fig. 1. Effect of Dex (50, 125 and 250 μg/100 g BW) administered to rats on TRβ$_1$ in liver tissue. (A) Representative Western blot: 40 μg of total protein from liver nuclear extracts were electrophorezed in 8% SDS/PAGE and transferred to nitrocellulose. Membranes were incubated with polyclonal TR antibody and HRP-secondary antibody. Bands were revealed by chemiluminescence. The membranes were stripped and reprobed with anti-actin antibody. (B) Densitometric analysis of Western blots. Data (mean ± SEM) are given as a percentage of means obtained in animals treated with vehicle (control group). Six samples were analyzed from each individual animal in each group in two separate experiments. *$p<0.001$ compared with the control group by one-way ANOVA followed by Student–Newman–Keuls test.

75% over control, respectively). However, the minor dose of Dex injected (50 μg/100 g BW) did not induced a significant modification in the expression of liver TRβ$_1$ mRNA.

Dex increased the transcriptional rate of rat liver TR gene

Nuclear run-on assay was used to examine whether the Dex-induced increase in TR mRNA was due to an increase in the transcriptional rate of the TR gene. As shown in Fig. 3A and B, Dex treatment (250 μg/100 g BW for 48 h) increased the transcriptional rate of liver TR gene by 255% over control value (arbitrary units, mean ± SEM, control: 0.18 ± 0.09; Dex treated: 0.46 ± 0.02 $p \leq 0.05$). These results indicated that Dex treatment increase TR expression through a mechanism that involves, at least in part, an enhanced transcriptional rate of TR gene. Considering that Dex increased TR mRNA by 75% (Fig. 2), an effect of Dex on mRNA degradation is not discarded.

Dex enhanced TRβ$_1$ promoter activity

Since Dex induced an increase of the transcriptional rate of TR gene, we next examined TRβ$_1$ promoter activity in response to Dex. For this purpose, COS-7 cells were transfected with the pGL-2-TRβ$_1$ promoter/luciferase (Luc). Because of the scarcely expressed TR and GR by COS-7 cells (Suzuki et al., 1994; Giguere et al., 1986), TRβ$_1$ and GR

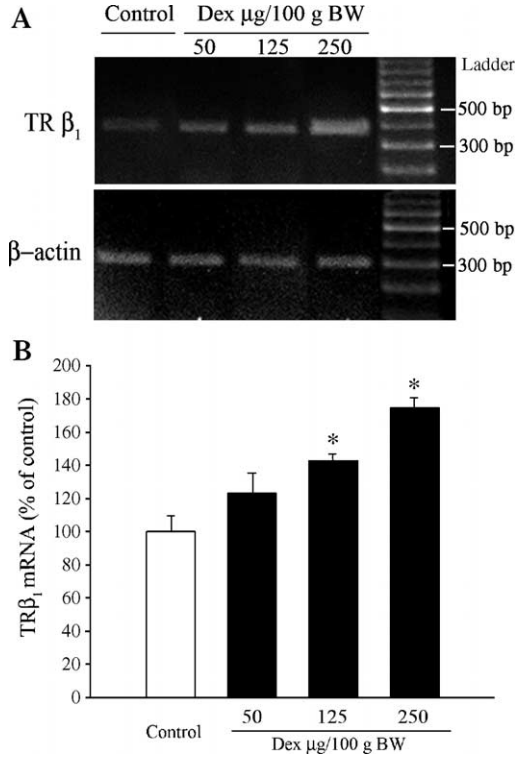

Fig. 2. Effect of Dex (50, 125 and 250 µg/100 g BW) administered to rats on $TR\beta_1$ mRNA expression in liver tissue. (A) RT-PCR: 1 µg total RNA was subjected to RT-PCR reaction for $TR\beta_1$ mRNA (upper panel) and β-actin (lower panel). (B) Densitometric analysis of RT-PCR. Data are expressed as the ratio between the densitometry of each $TR\beta_1$-specific signal and that of β-actin signal in the same sample. Values (mean ± SD) are given as the percentage of the control group. Data are from a representative experiment from a total of three with similar results. Four samples were analyzed from each individual animal in each group. $^{*}p < 0.01$ compared with the control group by one-way ANOVA followed by Student–Newman–Keuls test.

expression vectors were also cotransfected. Twenty four hours after transient transfection, cells were treated with or without T_3 and Dex for 12 or 24 h. At the end of this period, Luc activity was assayed in total cellular protein extracts. As previous reports (Suzuki et al., 1994) indicated that T_3 induced a significant increment of $TR\beta_1$ promoter activity under similar experimental conditions consistent with the presence of TRE in the $TR\beta_1$ promoter, T_3 was added as a control of the system and also to evaluate possible additive effects of T_3 and Dex.

As shown in Fig. 4, T_3 (10^{-7} M) treatment for 24 h increased $TR\beta_1$ promoter activity about 2.5 fold ($^{\#\#}p < 0.0005$) while no significant change was observed when cells were exposed to T_3 during 12 h. Addition of Dex (10^{-9} and 10^{-8} M) for 12 h resulted in a significant increase of the transcriptional activity only in the presence of T_3 (2-fold over 10^{-7} M T_3). Dex exposure during 24 h induced a significant increment in $TR\beta_1$ promoter activity both in the absence and the presence of T_3.

Dex induced proteins with TR gene promoter binding properties

Detailed sequence analysis (Schug and Overton, 1997 TESS: Transcriptional element search system, URL: http://www.cbil.upenn.edu/tess) of the human $TR\beta_1$ promoter from nt −1325 to +44 relative to the transcription initiation site revealed two consensus sequences that match for a glucocorticoid receptor-binding half-site and here designed as *site 1* and *site 2*. *Site 1* (AGAAGA) is located at nt −840 to −835 and *site 2* (TCTTCT) at −667 to −662. To determine whether these sequences are involved in the effect of Dex on the $TR\beta_1$ promoter, electrophoretic mobility shift assays (EMSA) were performed using 26 mer oligonucleotides comprising sequences of *site 1* ($TR\beta_1$ −850/−824) and *site 2* ($TR\beta_1$ −678/−652). The 5′ and 3′ flanking regions of both *sites* in the oligonucleotides correspond to the sequence present in the $TR\beta_1$ promoter depicted in Fig. 5A. Fig. 5B illustrates results for the binding of proteins from liver nuclear extracts from control and Dex treated rats (250 µg/100 g BW for 48 h) to $TR\beta_1$ −850/−824 and $TR\beta_1$ −678/−652 oligonucleotides. Binding assays using the oligonucleotide comprising *site 2* ($TR\beta_1$ −678/−652) did not show significant change in the DNA/protein complexes formed with nuclear extracts from Dex-treated and control rats (Fig. 5B; left panel, lanes 2 and 4). On the other hand, the oligonucleotide comprising *site 1* ($TR\beta_1$ −850/−824) revealed a differential pattern in complexes formation between nuclear proteins from control and Dex-treated rats (Fig. 5B; right panel, lanes 2 and 4).

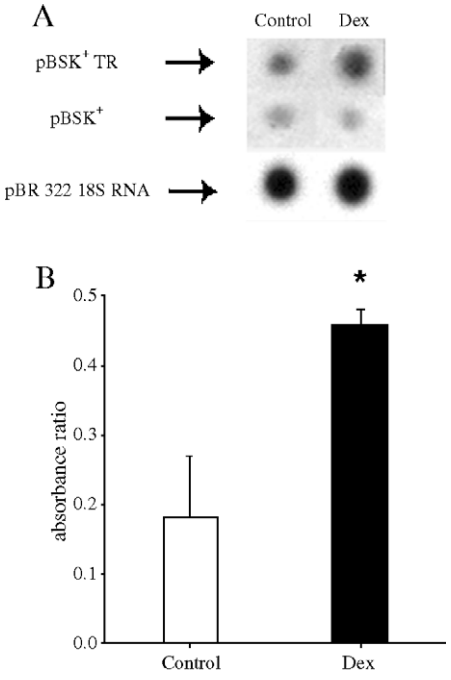

Fig. 3. Effect of Dex (250 µg/100 g BW every 12 h for 48 h) on the transcriptional rate of liver TR gene. (A) Representative run-on assay: 5×10^7 liver nuclei were submitted to each assay in the presence of GTP, ATP, CTP and a ^{32}P-UTP. Transcripts containing TR sequences were quantified by hybridization to nitrocellulose membranes containing immobilized TR cDNA ($pBSK^+$ $TR\beta_1$), $pBSK^+$ plasmid vector and pBR 322 18S rRNA. After hybridization, membranes were washed and autoradiographed. (B) Densitometric analysis of run-on assay: Data (mean ± SD) are given as the ratio between the absorbance of each TR-specific signal minus the signal of the vector alone and the absorbance of the 18S rRNA signal in the same sample. Data are from a representative experiment from a total of three with similar results. Three samples were analyzed from each individual animal in each group. $^{*}p < 0.05$ compared with the control group by Student *t* test.

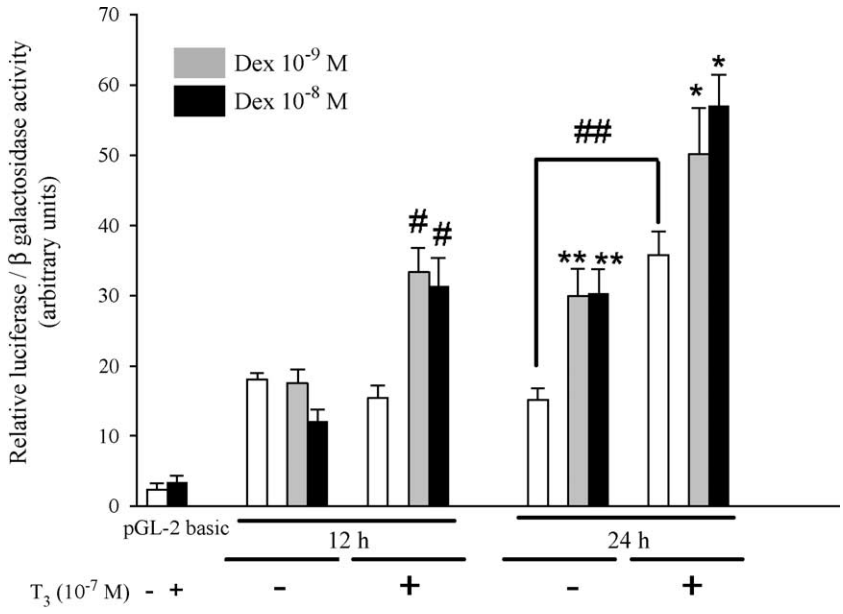

Fig. 4. Effect of Dex on TRβ1 promoter activity in COS-7 cells. The plasmid pGL-2 TRβ1 promoter (−1325 to +44/luciferase) (5 μg) was cotransfected to COS-7 cells with expression vectors for TRβ1 (1 μg) and GR (1 μg). Expression vector for β-galactosidase was also cotransfected to normalize the transfection efficiency. Luciferase and β-galactosidase activities were measured in cellular extracts 12 or 24 h after incubation with or without T_3 (10^{-7} M) and/or Dex (10^{-9} or 10^{-8} M). The promoterless pGL2-basic was transfected as a negative control. Values represent the means ± SEM of four experiments performed in triplicate. $^{\#}p<0.01$ vs. $+T_3$ 12 h; $^{*}p<0.05$ vs. $+T_3$ 24 h; $^{**}p<0.01$ vs. $-T_3$ 24 h; $^{\#\#}p<0.001$ $-T_3$ vs. $+T_3$ 24 h (ANOVA followed by Student–Newman–Keuls test).

Three complexes were formed (A, B and C) between the TRβ1 −850/−824 oligonucleotide and nuclear extracts from Dex-treated rats (lane 4) while a single complex (A) was visualized when nuclear extracts from control rats were incubated with the same oligonucleotide (lane 2). The retarded bands obtained were due to specific DNA binding as incubation of the same nuclear extracts with 100-fold excess of unlabeled oligonucleotide diminished them (lanes 3 and 5, left and right panel). To investigate the involvement of GR in complexes B and C formed between TRβ1 −850/−824 and nuclear proteins from Dex-treated rats, the binding reaction was assayed in the absence or presence of an anti-GR antibody. This antibody recognizes a GR DNA-binding domain epitope (GR M-20) and competed off binding to TRβ1 −850/−824 elements, revealing the involvement of GR in these binding complexes (Fig. 5C, lane 5). On the other hand, an anti-RXR used as a non-specific control antibody did not interfere the formation of the complexes (Fig. 5C, lane 6), indicating the specificity of the GR involvement.

Gel shift analysis of TRβ1 −850/−824 and protein extracts from COS-7 cells over-expressing GR (COS-7/GR) as depicted in Fig. 5D revealed the presence of two DNA–protein complexes with an electrophoretic mobility similar to the complexes B and C formed with nuclear extracts from Dex-treated rats (compare lanes 2 and 6). To verify whether the binding activity from COS-7/GR cells reflected a specific interaction between the GR and the radiolabeled probe, we performed EMSA using an antibody against GR. Results in Fig. 5D showed that the DNA–protein complexes were partially inhibited by the anti-GR antibody but not by a non-related antibody (compare lanes 7 and 8), thus identifying GR as the major component of both complexes.

To further analyze the specificity of the complexes involved in the protein–DNA interactions, we performed EMSA using mutated and GRE consensus oligonucleotides as competitors. Sequences of competitor oligonucleotides are outlined in Fig. 5A. Results depicted in Fig. 5E indicate that the complex A formed by the oligonucleotide TRβ1 −850/−824 and nuclear extracts from control and Dex-treated rats was effectively displaced by an excess of cold oligonucleotide (lanes 3 and 9) and by Mut A (lanes 4 and 10) but not by an excess of cold Mut B oligonucleotide (lanes 5 and 11). The formation of complexes B and C with nuclear extracts from Dex-treated rats were inhibited in the presence of the oligonucleotide TRβ1 −850/−824 (lane 9) or Mut A (lane 10) as competitors and partially inhibited after competition with the GRE consensus oligonucleotide (lane 12). On the contrary, an excess of Mut B was not able to inhibit the formation of both complexes (lane 11). Non-related DNA from salmon sperm at 100-fold excess had no effect on the shifted bands (lanes 7 and 13).

Discussion

The present study demonstrated that the administration of Dex to adrenalectomized rats resulted in an increase of liver TRβ1 protein, the main TR isoform in this tissue (Zhang et al., 2002). Since the magnitude of the cellular response to thyroid hormone depends on the abundance and/or affinity of the nuclear TR, it is possible that physiologically or pharmacologically induced alterations in the number or affinity of TR may be able to modify the tissue response to T_3 (Hodin et al., 1990). Previous reports indicated that under several experimental conditions, modifications in the number of TR paralleled changes in some specific

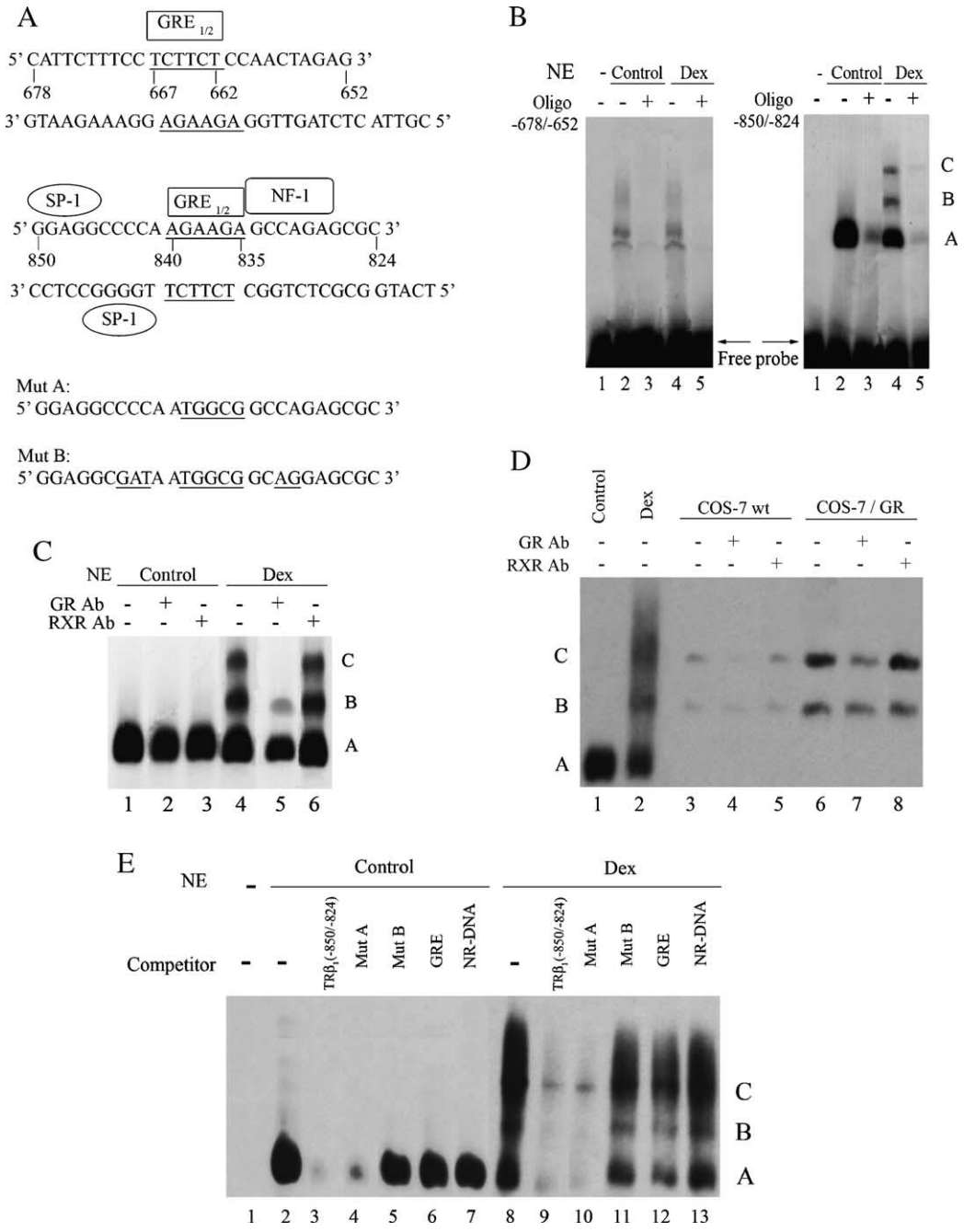

Fig. 5. (A) Diagram showing the nucleotide sequence of the −678 to −652 and −850 to −824 regions of the TRβ_1 promoter. The putative binding sites identified for different transcription factors in −850 to −824 sequences are also shown. The putative binding sites for the glucocorticoid receptor (GRE) are underlined. Sequences of mutated DNA oligonucleotides (Mut A and Mut B) used for competition in the gel shift assays are shown; mutated nt are underlined. (B) Electromobility shift analysis of TRβ_1 promoter sequences −850/−824 comprising *site 1* (left panel) and −678/−652 comprising *site 2* (right panel). EMSA were performed with 5 μg of protein nuclear extracts (NE) from liver of control rats (lanes 2–3, left and right panels) or Dex-treated (lanes 4–5, left and right panels) and ^{32}P-labeled double-stranded probe. Protein–DNA complexes were separated by nondenaturing polyacrylamide gel electrophoresis. In competition experiments a 100 times molar excess of the unlabeled oligonucleotide was used (lanes 3 and 5, left and right panels). (C) Electromobility shift analysis of TRβ_1 promoter sequences −850/−824 comprising *site 1*. EMSA were performed with 5 μg of protein nuclear extracts (NE) from liver of control rats (lanes 1–3) or Dex-treated (lanes 4–6) and ^{32}P-labeled double-stranded probe. Protein–DNA complexes were separated by nondenaturing polyacrylamide gel electrophoresis. Protein binding specificity was determined by preincubation of nuclear extracts with 2 μg of anti-GR (lanes 2 and 5) and 2 μg of anti-RXR (lanes 3 and 6). (D) Binding of protein extracts from COS-7 cells analyzed by EMSA. EMSA were performed with 15 μg of whole protein extracts from COS-7 wild type cells (COS-7 wt) (lanes 3–5) or COS-7 cells over-expressing GR (COS-7/GR) (lanes 6–8) and TRβ_1 −850/−824 ^{32}P-labeled double-stranded probe. Protein binding specificity was determined by preincubation of nuclear extracts with 2 μg of anti-GR (lanes 4 and 7) and 2 μg of anti-RXR (lanes 5 and 8). (E) Competition of the labeled TRβ_1 −850/−824 binding to nuclear extracts from liver of control and Dex-treated rats by 100-fold excess of the cold oligonucleotide as indicated. Lanes 2 and 8 indicate binding in the absence of a competitor. Consistent results were obtained in at least six experiments performed with different batches of protein and labeled probes.

tissue response to thyroid hormone (De Groot et al., 1977; Dillmann et al., 1978; Dillmann and Oppenheimer, 1979; Pellizas et al., 1998; Kaji and Hinkle, 1987). Moreover, TRβ_1 overexpression resulted in an increase in thyroid hormone responsiveness in mouse liver (Hayashi et al., 1996).

A correlation between the potentiation of T_3-induced metamorphosis in *Xenopus* and the enhancement of TR gene expression by glucocorticoids has been previously reported (Tata, 2000; Krain and Denver, 2004). Accordingly, the increased TR protein level induced by Dex in the liver of the rat could explain, at least in part, the increased specific metabolic response to T_3 seen in our previous studies (Recúpero et al., 1983, 1986). The augmented T_3-responsiveness by Dex requires de novo protein synthesis (Yamaguchi et al., 1999), a finding that suggested that Dex induced the expression of some factor(s) that enhanced the T_3-dependent transcriptional activation in hepatocytes. However, these authors were not able to detect an increase in TR level in cultured hepatocytes grown as spheroids treated with Dex.

A dissociation of TR maximal binding capacity and TR mRNA level in different tissues of adult rats suggests the participation of translational and/or posttranslational factors in the expression of the TR mRNAs (Strait et al., 1990; Tagami et al., 1993). However, physiological or pharmacological factors may also induce transcriptional and posttranscriptional modifications in the mRNA abundance that parallel the level of its protein (Huppart et al., 1993). This seems to be the case of the results obtained on the effect exerted by Dex, although the increase of TRβ_1 mRNA was detected by lower doses of Dex than TRβ_1 protein. On the other hand, results of nuclear run-on assay presented here indicated that Dex increased the transcriptional rate of the TR gene. This finding indicated that the Dex-enhanced TR expression could respond to a stimulatory action of Dex on TR mRNA transcriptional rate. Moreover, transient transfection assays using a reporter gene driven by the TRβ_1 gene promoter demonstrated that Dex increased the transcription of TRβ_1 gene. Nevertheless, an effect of Dex on TR mRNA stability could not be disregarded.

The enhancement of TRβ_1 promoter activity after 24 h of T_3 exposition in transfection assays (Fig. 4) was consistent with previous reports from Suzuki et al. (1994). The effect of T_3 on the TRβ_1 gene is expected to occur since consensus sites for TREs in the TRβ gene promoter have been previously identified both in human and *Xenopus* (Suzuki et al., 1994; Machuca et al., 1995). Dex induced a significant increment of TRβ_1 promoter activity after 12 h only when T_3 was present while the Dex-induced increase of the promoter activity after 24 h was observed both in the absence and the presence of T_3, although a higher stimulation was induced when T_3 was also included. Laudet et al. (1993) characterized a functional promoter in the human TRα gene that harbours a consensus sequence for a glucocorticoid response element (GRE). Moreover, the authors observed an increased transcriptional activity of TRα gene by Dex in HeLa cells, although a direct action of GR on this GRE was not investigated.

Glucocorticoids produce their effect on responsive cells either through a direct interaction of GR with the consequent binding to GRE in the regulatory regions of target genes or indirectly through de novo protein synthesis (Hayashi et al., 2004; Schoneveld et al., 2004). The oligonucleotide probe TRβ_1 −850/−824 which included a consensus sequence for a GRE half-site (*site 1*) present in the TRβ_1 gene promoter formed three complexes (A, B and C, Fig. 5B, right panel lane 4) when incubated with liver nuclear protein extracts from Dex-treated rats while a single complex (A) was observed with nuclear protein extracts from control animals (Fig. 5B, lane 2, right panel). This finding indicates that Dex treatment induced the binding of additional proteins related to or neighboring the DNA sequence of the GRE in the TRβ_1 promoter. The abundance of the oligonucleotide–protein complexes B and C decreased markedly after the preincubation with a specific anti-GR antibody suggesting that these complexes resulted from the interaction of GR with the synthetic oligonucleotide probe. Results of EMSA with protein extracts from COS-7 cells over-expressing GR and the oligonucleotide TRβ_1 −850/−824 revealed the presence of two complexes that migrated similarly to those seen with liver nuclear extracts from Dex-treated rats (Fig. 5 D, lane 6). The addition of an antibody against GR diminished both complexes (Fig. 5 D lane 7), strongly suggesting the involvement of GR in the additional complexes formed by the effect of Dex. Although GR levels are down-regulated by glucocorticoids (De Bosscher et al., 2003), once the ligand binds to GR, chaperones molecules dissociates allowing the nuclear localization of the activated GR-steroid complex, its binding to GRE and its interaction with coactivator complexes. Since the degree of GR nuclear localization is a critical step in determining the magnitude of GR function (Hayashi et al., 2004), the administration of Dex to rats would induce the translocation of GR to the nucleus and the consequent interaction with the TRβ_1 promoter.

The formation of the three DNA–protein complexes was similarly inhibited in the presence of either TRβ_1 −850/−824 oligonucleotide or mutated GRE (Mut A) competitors. The region −850/−824 of the TRβ_1 promoter contains putative sequences for the ubiquitous specificity protein 1 (SP-1) transcription factor and nuclear factor 1 (NF-1). Mutation of these elements in the Mut B oligonucleotide abolished the capacity to compete with TRβ_1 −850/−824 oligonucleotide. Therefore, it is tempting to speculate that the presence of GR was necessary for TRβ_1 −850/−824–proteins interactions albeit binding of other proteins flanking GRE sequences were also required. Binding of NF-1 has been shown to be essential for GR-mediated transactivation of the mouse mammary tumor virus (MMTV) promoter (Hebbar and Archer, 2003). On the other hand, SP-1 have been described to interact with GR and cooperate in the regulation of the expression of different genes (Lamas et al., 1997).

In spite of the similar nucleotide sequence of *site 1* and *site 2* (see Materials and methods and Fig. 5 A), the latter did not reveal differences in the complexes formed with nuclear extracts from control or Dex-treated rats. It is highly conceivable that the specificity of the GR binding to GRE is determined not only by the GRE DNA sequence, but also by the interaction of the GR with other transacting factors bound

to sequences flanking the GRE. Among the different types of binding sites for the direct interaction of GR with DNA, a GRE half-site involved in the activation of gene expression through the binding of GR as monomer has been described (Slater et al., 1993). Although in a number of genes the presence of a GRE half-site was sufficient to relay glucocorticoid signalling (Schoneveld et al., 2004), other authors were unable to reveal GRE half-sites acting as a simple element in activating transcription, a finding in consonance with our results. Instead, GRE half-sites seem to require additional elements to mediate a glucocorticoid response (Bristeau et al., 2001; Faust et al., 1996). A class of glucocorticoid response elements known as "composite GREs" or glucocorticoid response units "GRUs" has been proposed (Lamas et al., 1997; Kallwellis-Opara et al., 2003). Promoter analysis of Dex-regulated genes in lymphoid cell systems studied by microarray assays by Thompson et al. (2004) revealed that only a minority of these genes contained a classic palindrome response element for GR (classic GRE). Moreover, other genes were found to contain a GRE half-site. These authors concluded that a large proportion of genes induced by Dex increased their transcription from GR acting at GRU lacking a cognate GRE. Further studies are necessary to disclose whether other proteins are involved in the interaction of GR-GRE in the TRβ_1 promoter.

In conclusion, in this study we demonstrated that Dex induced an increment in liver TRβ_1 expression both at the mRNA and the protein level. The increased transcriptional rate of TR gene observed in vivo could explain, at least in part, the enhanced TRβ_1 expression. Further, the increased transcriptional activity of TRβ_1 promoter induced by Dex most probably result from the interaction of GR with a consensus site for GRE and the binding of other factors to GRE flanking sequences present in TRβ_1 promoter.

Since glucocorticoids are very often used clinically, especially to treat inflammatory and auto-immune diseases, the results obtained in the present study are of interest considering the impact of glucocorticoids administration on the peripheral thyroid hormone metabolic action.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Ciencia y Técnica (FONCYT), Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT), Agencia Córdoba Ciencia S.E. and Third World Academy of Sciences (TWAS). MMM, MLV and SS are Fellows at CONICET. CGP and AHC are established researchers at CONICET.

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