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ORIGINAL RESEARCH ARTICLE

Glucocorticoid receptor alpha and beta isoforms are not mutated in bipolar affective disorder

P Moutsatsou[1], A Tsolakidou[1], G Trikkas[2], C Troungos[1] and CE Sekeris[1,3]

[1]*Laboratory of Biological Chemistry, University of Athens Medical School, 75 Mikras Asias Str, GR-115 27 Goudi, Athens, Greece;* [2]*Eginition Hospital, Department of Psychiatry, University of Athens Medical School, 74 Vas Sofias Ave, Athens, Greece;* [3]*Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas Constantinou Avenue, GR-116 35 Athens, Greece*

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The periodically hyperactive hypothalamic-pituitary-adrenal (HPA) axis in bipolar affective disorders, as well as the reported changes in the binding characteristics of the glucocorticoid receptor (GR), suggest the possible involvement of the GR in the aetiopathology of this disease. This was investigated by screening the coding sequences of both GR isoforms, GRα and GRβ, for the presence of mutations. As a genetic predisposition has been implicated, we included in this study bipolar patients who were siblings. By RT-PCR of peripheral blood mononuclear cells from patients suffering from bipolar illness, using primers spanning the whole length of the GRα and GRβ coding region and subsequent agarose gel electrophoresis, heteroduplex and sequence analyses, no GR mutations could be detected. Since glucocorticoid receptor activity can be modulated by agents other than the respective ligand (eg by growth factors, cytokines and stress signals), our results favor derangements in the modulation of GR activity by such agents and not in the primary structure of the receptor as aetiopathologic factors of bipolar disease. *Molecular Psychiatry* (2000) **5,** 196–202.

Bipolar affective disorder and major depression are considered stress-related diseases. A genetic predisposition has been implicated; stressful life events, however, are necessary to trigger the onset of these diseases.[1–3] An hyperactive hypothalamic-pituitary-adrenal (HPA) axis, serotonin depletion and a stimulation of the sympathoadrenal system, reflecting central nervous system stimulation, are consistent findings in depression.[4–6] Moreover, there is a loss of circadian rhythm in the HPA axis function[7] and a loss of sensitivity to glucocorticoids, as shown by the lack of response in the dexamethasone suppression test.[8,9] Antidepressant therapy normalizes HPA axis activity and catecholamine secretion, restores the regulatory ability of glucocorticoids through the feedback mechanism,[10,11] thus supporting signs and symptoms being 'state dependent' in this disease.

The glucocorticoid receptor (GR) has for long been considered a primary factor in the aetiology of depression. The hippocampus and hypothalamus are rich in GR and are also the sites responsible for the regulation of the circadian rhythm and the negative feedback mechanism, therefore a defect in the number and/or function of GR could be a causative factor for disturbances seen in depression.[12]

Many studies concerning the binding characteristics of GR (number of binding sites and affinity constant) in human peripheral blood mononuclear cells of depressed patients, compared to normal individuals, reported results that are controversial and inconclusive.[13–17] However, no data on the molecular structure of the GR in human bipolar illness have been reported. The molecular structure of the GR and its gene have been fully elucidated[18–20] and important insights have been obtained on the molecular mode of action of GR by way of modulation of transcription and interaction (cross-talk) with other key regulatory molecules.[21–25]

Mutations of GR at phosphorylation sites clustering in the N-terminal transcriptional regulatory domain or hormone binding domain have been reported to result in enhanced or diminished sensitivity to glucocorticoids.[26–28] Thus, mutations of the glucocorticoid receptor, particularly on sites involved in the cross-talk of GR with other regulatory systems (such as nervous system), could alter GR activity and could explain the 'state-dependent' dysregulation of the HPA axis activity present only during depressive episodes.

Moreover, a new isoform of the glucocorticoid receptor, GRβ, resulting from alternative splicing of the hGRα pre-mRNA, has been described, which does not bind glucocorticoids and by heterodimerization with GRα exerts a dominant-negative effect on GRα action, thus affecting tissue sensitivity to glucocorticoids.[29] On the basis of the above, we decided to investigate the possible presence of mutations in the GRα and GRβ coding region in patients with bipolar illness by use of RT-PCR, heteroduplex and sequence analyses.

We provide evidence that no such GR mutations could be detected and maintain that defects in post-translational modification of GR (such as phosphorylation) evoked possibly by stress signals, could alter GR activity and may be the aetiopathologic factor of bipolar disease.

We have submitted to RT-PCR analysis and agarose gel electrophoresis, RNA isolated from peripheral blood mononuclear cells of 12 normal individuals and 15 patients suffering from bipolar affective disorder I

or II, according to DSM-IV criteria. The patients were under drug treatment in various phases of the illness (Table 1). For comparison, we similarly analysed samples from healthy individuals without a personal or family history of psychiatric illness. We have used six pairs of primers spanning the whole length of GRα, plus one extra primer to cover the whole length of GRβ. The results shown in Figure 1 indicate that both full-length wild-type GRα and GRβ are expressed in PBMC obtained from normal individuals and from patients.

Using the primer pair D, a lower mobility band (LMB), in addition to the main band, was detected in the preparations from both normal and depressed patients, of still unknown structure. This PCR product, containing both the main GR band and the LMB, was submitted to RFLP analysis using the restriction enzyme *Eco*RI which cleaves at 1628 bp of the GR gene coding region. The restriction pattern was the one corresponding to GR, as the amplified product (364 bp) was cleaved into two fragments of 276 bp and 88 bp, as expected for GR. The LMB gave also an 88-bp fragment and another fragment (LMBf) (results not shown) of slightly lower mobility than the 276-bp fragment. These results suggest that the lower mobility band is a GR-variant whose detailed structure and biological role remain to be elucidated.

All the PCR products were submitted to analysis for the detection of mutations, using the heteroduplex technique (Figure 2). The results depicted in Figure 2 reveal that no mutations can be detected in the preparations either derived from normal or from bipolar patients. The bands Ha and Hb (Figure 2D) correspond to heteroduplexes formed between the wild-type GR fragment and the LMB fragment which are present in the PCR product using primer pair D.

An underlying concept of the study was that if GRα gene alterations play a major role in the determination of genetically linked bipolar patients, mutations or polymorphisms should be shared more commonly than expected by chance among these family pairs. Therefore, sequence analysis was carried out in six patients who were siblings. The results obtained documented the absence of mutation or polymorphism of the GRα gene.

In a recent study of an elderly population,[30] five polymorphisms in the glucocorticoid receptor gene were identified, but they were not associated with glucocorticoid resistance, observed in this population, as diagnosed by the use of an overnight dexamethasone suppression test. Furthermore, a polymorphic variant of GR, with a change of an Asparagine to Serine at codon 363, has been reported.[31] In the present work we were unable to detect these polymorphisms, possibly due to their low frequency (less than 5%).

The molecular action of glucocorticoids involves a series of protein factors, such as heat shock proteins (HSPs), transcription factors (AP-1, NFκB-Rel), histone acetyl transferases and others.[22] Shimizu *et al*[32] described a 29-bp deletion in the 5′ non coding region and a 133-bp deletion in the coding sequence of HSP70 mRNA in depressed, but not in normal individuals. It is worthwile to mention that altered patterns of the transcription factor AP-1 have been reported in asthma suggesting disturbed intracellular signalling and have been linked to glucocorticoid sensitivity or resistance observed in this disease.[33] Therefore, the possibility that transcription factors or genes for other proteins, crucial for the expression of GR activity, may be affected in bipolar illness could be an important determinant in the pathophysiology of depression.

As the relative levels of GRα to GRβ (at the protein or the mRNA level) could be a determinant of glucocorticoid sensitivity, a quantitative study of GR or its mRNA, applying Western Blot analysis or quantitative PCR, respectively, warrants further investigation.

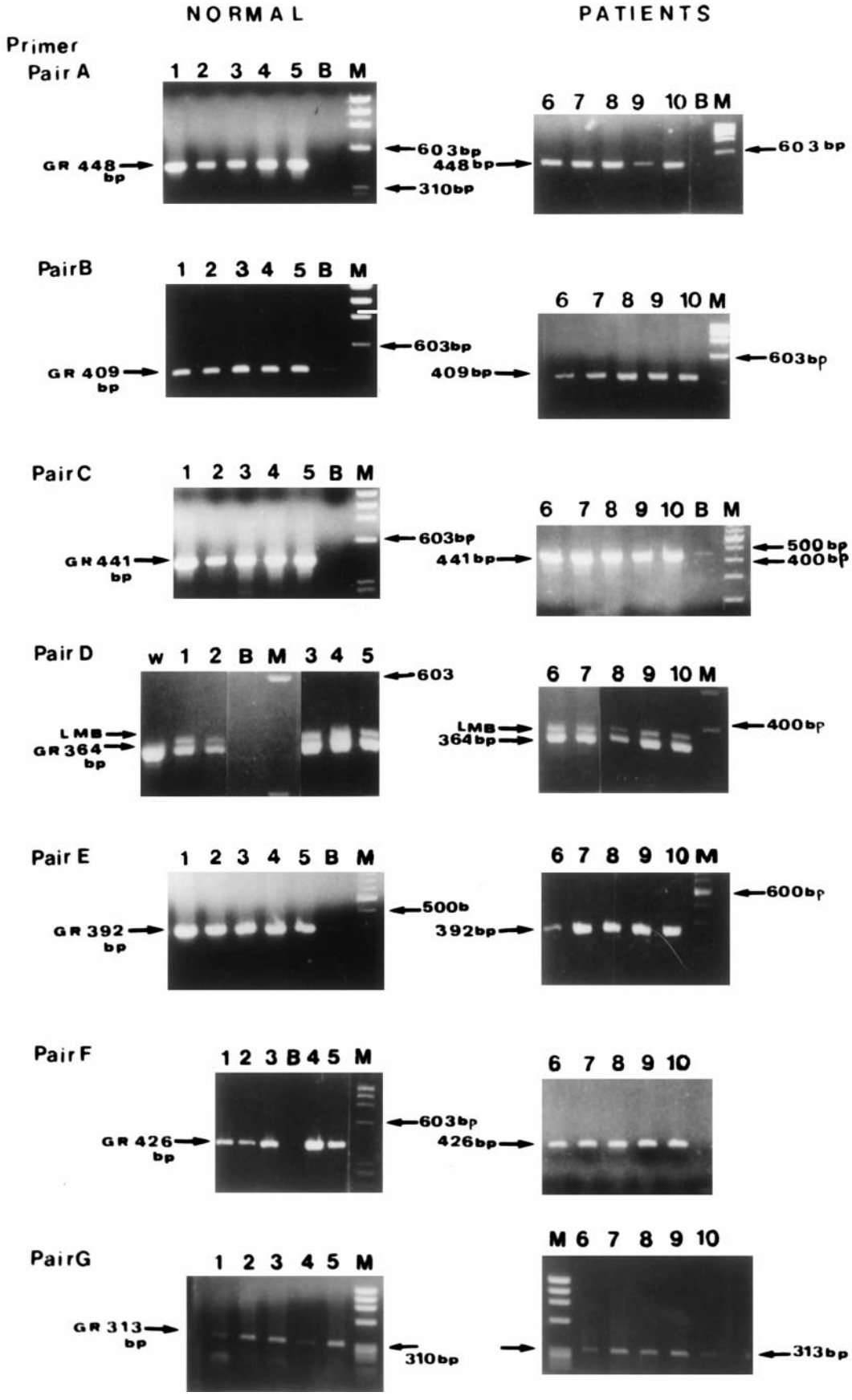
Phosphorylation of the GR represents a major post-translational modification,[34] induced by the steroid ligand but also by cytokines, growth factors, stress signals and other regulatory molecules, by way of multiple kinases, which either positively or negatively affect receptor evoked transcriptional enhancement, indicating that a wide spectrum of regulatory inputs could modulate, in conjunction with the steroid hormones, the function of GR.[24,25] Genetic defects in such

Table 1 Demographic and clinical characteristics of the patients

No.	*Name*	*Gender*	*Age (years)*	*Education (years)*	*Diagnosis*	*Mood state*
1	TE	F	69	6	BAD I	D
2	AA	F	44	12	BAD I	D
3	AG	M	70	12	BAD I	N
4	AJ	M	66	16	BAD II	N
5	KM	F	55	12	BAD I	N
6	KA	F	72	12	BAD I	N
7	LV	F	50	6	BAD II	N
8	PV	F	50	14	BAD I	N
9	SA	F	39	14	BAD I	N
10	KD	A	59	14	BAD I	D
11	HI	F	35	10	BAD I	N
12	MA	F	40	12	BAD I	N
13	VE	M	63	16	BAD I	M
14	PM	F	58	12	BAD I	HM
15	KV	F	49	16	BAD I	D

BAD I or II: bipolar affective disorder I or II; D: depression; N: normothymia; M: mania; HM: hypomania.

Figure 1 RT-PCR products of wild-type full length GR (GR) from peripheral blood mononuclear cells of normal individuals (1–5) and of patients with bipolar affective disorder (6–10). Total RNA was obtained and amplified using specific primers. Primer pairs A–F were used to amplify fragments of hGRα cDNA, whereas primer pair G was used to amplify a fragment specific to the hGRβ cDNA sequence. PCR products were submitted to electrophoresis in 3% agarose gel and visualized after staining with ethidium bromide. Arrows point to the expected size of PCR products (GR) as well as to the lower mobility band (LMB). Results shown are representative of 12 different normal individuals and 15 patients. M = marker, B = blank, W = GRα cDNA.


NORMAL
PATIENTS
Primer
Pair A
1 2 3 4 5 B M
GR 448 bp
603bp
448bp
310bp
6 7 8 9 10 B M
603 bp
PairB
1 2 3 4 5 B M
GR 409 bp
603bp
409bp
6 7 8 9 10 M
603bp
PairC
1 2 3 4 5 B M
GR 441 bp
603bp
441bp
6 7 8 9 10 B M
500 bp
400 bp
Pair D
w 1 2 B M 3 4 5
603
LMB
GR 364 bp
6 7 8 9 10 M
LMB
364bp
400bp
Pair E
1 2 3 4 5 B M
GR 392 bp
500b
392bp
6 7 8 9 10 M
600bp
PairF
1 2 3 B 4 5 M
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PairG
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GR 313 bp
310bp
M 6 7 8 9 10
313bp

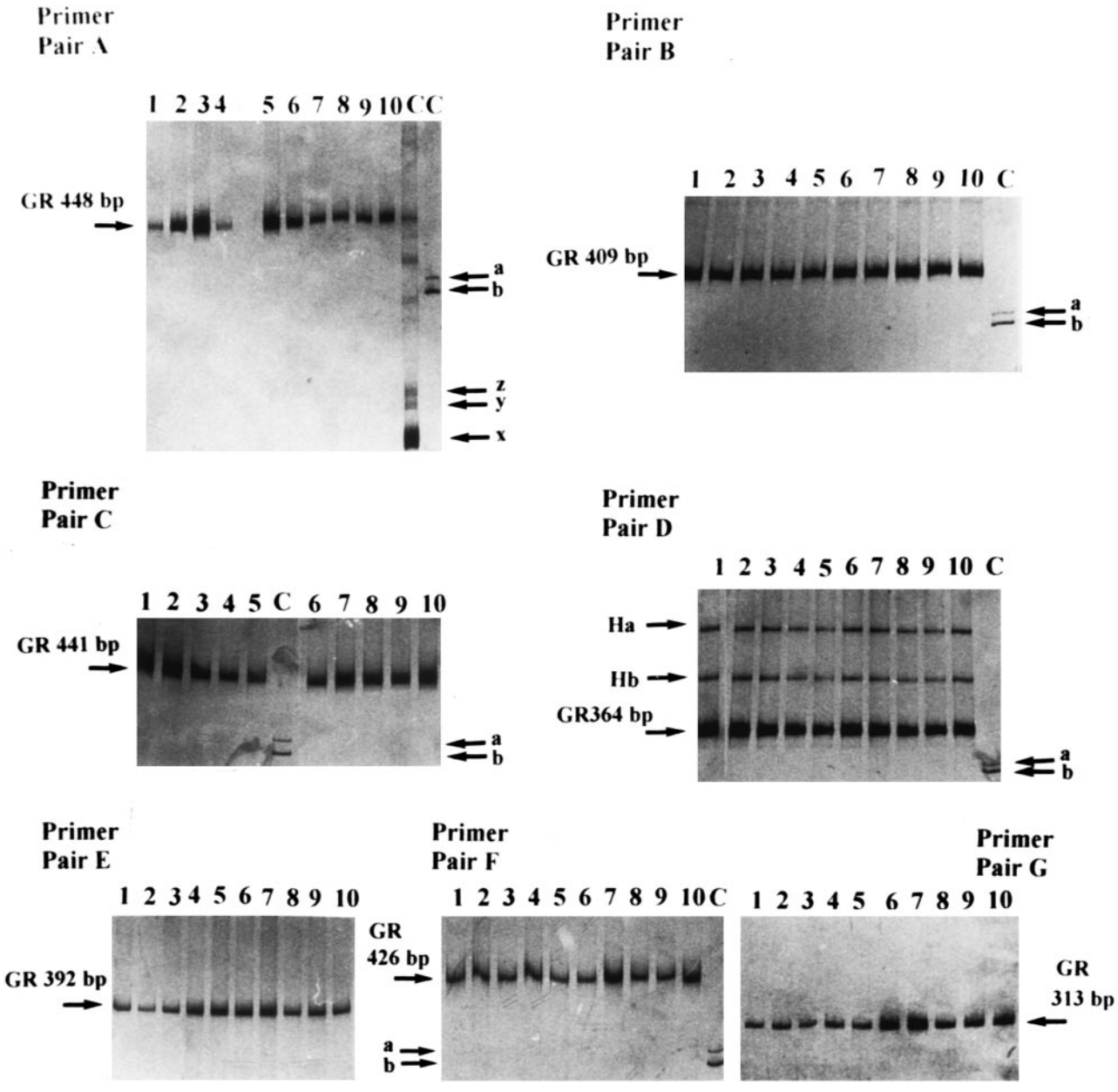

Figure 2 Heteroduplex analysis. Total RNA was obtained from normal individuals (1–5) and patients affected by bipolar illness (6–10) and amplified using specific primers (A–G). PCR products were submitted to heteroduplex analysis using MDE polyacrylamide gels and GR cDNA fragments were visualized after silver staining. Arrows point to the expected GR homoduplex molecules. C = commercial control sample containing a mixture of homoduplex (b) and heteroduplex molecules (a). C′ = In house control sample of known mutation submitted to heteroduplex analysis. A mixture of homoduplex molecules (x) and heteroduplex molecules (y, z) were obtained. Heteroduplex analysis of Ha, Hb = GR cDNA fragment and lower mobility band (LMB) obtained after PCR-amplification using primer pair D, gave heteroduplex molecules Ha and Hb.

enzymatic proteins, involved in signal transduction, crucial for the receptor's function, could be a key factor in the pathophysiology of bipolar illness and a future research target in the study of depression.

Moreover, an imbalance between neural signals (known to be altered in depressed patients) and corticosteroids, both modulating glucocorticoid receptor transcriptional activity by way of posttranslational modification, could be a causative factor altering GR function during depressive episodes. Such a concept could possibly explain the 'transient state' of hypercortisolaemia and the loss of sensitivity to glucocorticoids observed in the depressive phase. The use of serotonin reuptake inhibitors as antidepressant therapy restoring HPA axis dysregulation, as well as reports from an earlier work[35] in which increased serotonin availability

augmented cortisol-induced feedback inhibition in humans, is supportive of this concept.

Methods

Patient data

Fifteen patients (four males and 11 females) suffering from bipolar I or II affective disorders, according to DSM-IV criteria for mental illnesses, and 12 normal control subjects (six males and six females), sharing the same sociodemographic characteristics, participated in this study. All patients attended the Outpatients' Clinic of Athens University Department of Psychiatry, whereas the controls were laboratory personnel (Athens University, Biological Chemistry Department), with no personal or family psychiatric history. The age range of patients was 39–72 years and that of controls 24–45 years. Table 1 shows the main demographic and clinical characteristics of the patients. Among the 15 patients, three couples were siblings (Nos. 3 and 4, 5 and 6, 13 and 14). All patients were under drug treatment, such as lithium or carbamazepine and phenothiazines. The patients' mood state was normothymic (nine patients), depressed (four patients) and manic (two patients). All subjects gave their consent that the material be used for research purposes.

Sample collection

Venous blood (20 ml) was collected into precooled heparinized vacutainers, placed immediately on ice and peripheral blood mononuclear cells were isolated by Ficoll Hypaque gradient centrifugation. The number of cells as well as their viability was determined. Cellular RNA was isolated from PBMC according to a direct RNA extraction protocol (QIAGEN 'Rneasy Protocol for isolation of total RNA from Tissues and Eukaryotic Cells'). The concentration, total yield and purity of RNA was determined spectrophotometrically by measuring absorbance at 260 nm and 280 nm (ratio A_{260}: A_{280} = 1.8–2.0).

Reverse transcription-PCR amplification

Total RNA (0.5 μg per reaction) denatured at 70°C for 3 min, RNasin (20 IU per reaction, Promega, Madison, WI, USA) was added and subsequently reverse transcribed in a final volume of 20 μl made with the reaction buffer supplied with the M-MLV reverse transcriptase (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT final concentrations) in the presence of M-MLV reverse transcriptase (200 units per reaction, Moloney Murine Leukemia Virus Reverse Transcriptase, Promega), random hexamers (0.5 μg per reaction, Promega) and dNTPs (final concentration 1 mM each). The reaction was allowed to proceed for 60 min at 37°C, terminated by heating at 95°C for 5 min and the preparation placed immediately on ice.

The total reaction mixture (20 μl) was submitted to PCR amplification by using seven pairs of primers, specific to the human glucocorticoid receptor gene coding region (hGRa cDNA and hGRβ cDNA) (Figure 3), which are the following: Primer pair A (map position 125–573, 5′ATTCACTGATGGACTCCAAAGAATCA-3′, 5′-CACAGCAGTGGATGCTGAACTCTTGG-3′); primer pair B (map position 547–954, 5′-CCAAGAGTTCA GCATCCACTGCTGTG-3′, 5′-ACTTGGGGCAGTGTTA CATTACT-3′); primer pair C (map position 936–1376,

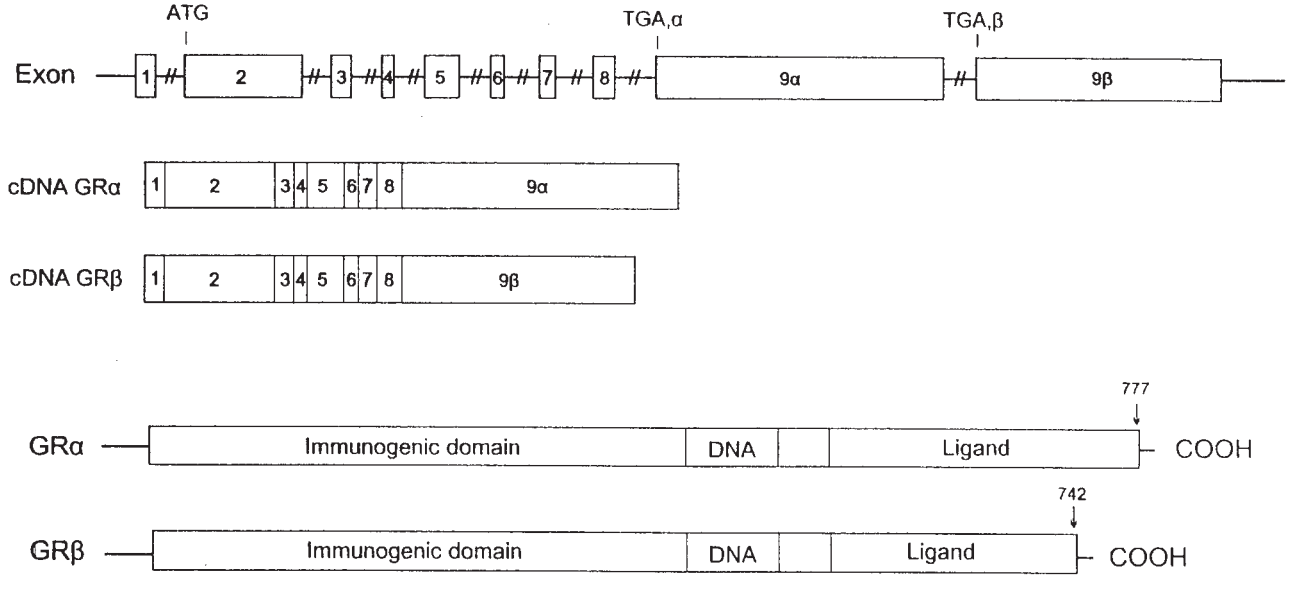

Figure 3 Schematic representation of human GR cDNA consisting of 10 exons (open boxes). Human GRα cDNA contains exon 9α (in addition to the first eight exons) and human GRβ cDNA contains exon 9β. Exon 1 is a non coding sequence, exon 2 encodes for the N-terminal domain of the GR protein molecule, exon 3 and exon 4 for the DNA-binding domain, and exon 5–exon 9a for the hormone-binding domain. The two isoforms have the first 727 NH_2-terminal aminoacids in common, so they both contain the transactivation and the DNA-binding domains. hGRβ contains in its COOH-terminus a unique 15-aminoacid sequence in place of the last 50 aminoacids of the hGRα isoform, does not bind glucocorticoid hormones and is transcriptionally inactive.

5′-CAGTAATGTAACACTGCCCCAAGT-3′, 5′-CCTG TTGTTGCTGTTGAGGAGCTGG-3′); primer pair D (map position 1352–1716, 5′-CCAGCTCCTCAACA GCAACAACAGG-3′, 5′-GAGTTGTGGTAACGTTGC AGGAC-3′); primer pair E (map position 1693–2070, 5′-GTTCCTGCAACGTTACCACAACTC-3′, 5′-CATG TGTTTACATTGGTCGTACATGC-3′); primer pair F (map position 2045–2471, 5′-GCATGTACGACCAAT GTAAAC ACATG-3′, 5′-GGCAGTCACTTTTGATGAA ACAGAAG-3′); primer pair G (map position 2045–2358, 5′-GCATGTACGACCAATGTAAACACATG-3′, 5′-GAT TAATGTGTGAGATGTGCTTTCTG-3′) (numbering of nucleotides according to Hollenberg *et al*[18]). The PCR reaction volume (100 μl) contained 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, dNTPs (final concentration/200 μM each), 1 μl of each primer (4 ng μl^{-1}) and 0.3 IU Taq polymerase (Promega). The PCR conditions were: 35 cycles of 2 min at 94°C (denaturation), 1.5 min at 63°C (annealing), 1 min at 72°C (extension), followed by one cycle at 72°C for 10 min and then stored at 4°C.

In each PCR experiment a tube without template was included, to check for contamination by extraneous genomic DNA or cDNA. Another tube containing plasmid GRα cDNA or GRβ cDNA was also included in each amplification experiment to control PCR specificity conditions. Each pair of primers used was compared to GeneBank sequence libraries to assure specific amplification of human GR cDNA. PCR products (10 μl) were submitted to electrophoresis on a 3% agarose gel. After staining with ethidium bromide they were evaluated by comparison to reference size markers ΦX174 DNA, *Hae*III (Promega) and to 100-bp DNA ladder (Gibco BRL, Gaithersburg, MD, USA). Restriction fragment length polymorphism (RFLP) using the enzyme *Eco*RI (Promega) was carried out to elucidate the nature of a faint band with slightly lower mobility (LMB) than the wild-type GR band obtained by using the primer pair D.

*Eco*RI digestion was carried out in a total volume of 50 μl: PCR products (15 μl) were mixed with the appropriate buffer (5 μl) (2.5 mM pH 7.8, 10 mM potassium acetate, 1 mM magnesium acetate, 0.1 mM DTT final concentration, Promega) in the presence of bovine serum albumin (0.5 μl, final concentration 100 ng μl^{-1}), H_2O (27.5 μl) and *Eco*RI (20–24 IU).

Heteroduplex analysis

The PCR product from patient samples (2 μl) was mixed with an equal volume of the PCR product from a normal sample heated for 4 min at 95°C, subsequently left to attain room temperature and then subjected to heteroduplex analysis using MDE (mutation detection enhancement) polyacrylamide gels (FMC) (1 × MDE, 0.6 × TBE, 0.6 × APS (10%), 0.06 × TEMED final concentration). The samples (4 μl) were submitted to electrophoresis for 20 h at 650 V and cDNA fragments were visualized after silver staining. In each heteroduplex analysis experiment a tube containing a known heteroduplex mixture (heteroduplex control, Promega) as well as an in-house control sample were included to serve as control and to assure proper heteroduplex analysis.

Sequence analysis of PCR products

The PCR products spanning the whole length of GRα from six patients (three couples who were siblings; 3 and 4, 5 and 6, 13 and 14, Table 1) were sequenced as follows. The purified (by use of Qiaquick spin kit, Qiagen) PCR products (500 ng) were amplified in a sequencing reaction volume (20 μl) containing the appropriate labelled primers, buffer and Taq polymerase. Amplification was performed on Perkin Elmer 9600 (95°C for 2 min, and 95°C for 30 s, 52°C for 25 cycles). Stop solution was added and denaturation (92°C for 2 min) followed before loading on a denaturating sequencing gel (42% w/v urea, 0.6 × TBE and 6% Long Ranger gel solution FMC). The gel was run on ALF express Pharmacia Automated Sequencer for 800 min at 1500 V at 55°C.

The sequencing procedure as described above was carried out in Microchemistry Lab, Foundation for Research and Technology Hellas (FORTH, Greece), PO Box 1527, Herakleion 71110 Crete, Greece.

Accession number

The numbering of nucleotides for the GRα and GRβ gene coding region was based on accession numbers M10901 and M11050, respectively.

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Correspondence: P Moutsatsou, University of Athens Medical School, Department of Biological Chemistry, 75 Mikras Asias Str, GR-115 27 Athens, Greece. E-mail: biolchem@ath.forthnet.gr
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