Stimulation of transcription in vitro from a liver-specific promoter by human glucocorticoid receptor (hGRα)

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The rat tyrosine aminotransferase (TAT) gene is a liver-specific and glucocorticoid-inducible gene. Previous studies have shown that the TAT promoter (TATα35; nt −350 to +1) is able to sustain liver-specific gene expression both in transient transfection and in a transcription assay in vitro [Schweizer-Goyer, Groyer, Cadepond, Grange, Baulieu and Pictet (1994) Nucleic Acids Res. 22, 1583–1592]. Here we report that the basal transcriptional activity generated from TATα35 in the presence of crude liver nuclear extracts is enhanced by added human glucocorticoid receptor (hGRα), provided that TATα35 sequences were flanked (5′) with a glucocorticoid responsive unit (GREII of the TAT gene, including its 5′-CCAAT flanking sequence). Two sources of hGRα were used: nuclear extracts prepared from SI9 insect (SI9-NEs) cells over-expressing hGRα, and hGRα from pRShGRα-transfected COS-7 cells, enriched by high-performance ion-exchange chromatography. The enhancement of transcription in vitro (1.5–4.5-fold) was dependent on the amount of added hGRα and independent of the nature (agonist or antagonist) of the ligand. Moreover, the hGRα-mediated stimulation of transcription was (i) dependent on GRE/PRE but not by a 100-fold excess of oestrogen response element and (ii) receptor-dependent (SI9-NEs prepared from uninfected SI9 cells or from SI9 cells infected with wild-type baculoviral DNA did not enhance transcription). Taken together, these experiments support the conclusions that in vitro the glucocorticoid receptor is able to enhance transcription from genomic, liver-specific, promoter sequences (those of the TAT gene), and that this enhancement of transcription from the liver-specific TATα35 promoter is dependent both on the glucocorticoid receptor and on the latter’s interaction with its cognate response elements.

INTRODUCTION

The synthesis and activity of the enzymes that participate in gluconeogenesis are subjected to complex regulatory mechanisms involving tissue-specific as well as hormonal signals. The gene encoding the gluconeogenic enzyme tyrosine aminotransferase (TAT) is switched on in the parenchymal cells of the liver during the neonatal period [1], liver-specific expression being (i) dependent on both the promoter [2,3] and remote enhancer elements [4] and (ii) modulated by environmental cues (induced by glucocorticoids and glucagon via its intracellular mediator cAMP and repressed by insulin) at the transcriptional level [5–7].

Both constitutive and hormone-dependent TAT regulatory sequences (promoter and enhancers) appear as modular transcriptional control units where ubiquitous (nuclear factor-Y, glucocorticoid receptor, cAMP responsive element binding protein) and liver-specific (hepatic nuclear factor 3, hepatic nuclear factor 4, nuclear factor 1liv, and to a smaller degree hepatic nuclear factor 1) trans-acting factors synergize [2,3,8–10].

The molecular mechanisms underlying the activation of gene transcription and the interaction(s) between gene regulatory factors have been, and are currently being, thoroughly studied. In this connection, transcription in vitro has proved to be a powerful tool in the experimental analysis of the tissue-specific activity of genomic promoter sequences (e.g. albumin, pyruvate kinase and TAT), each consisting of a modular transcriptional unit composed of a unique array of cis elements for both ubiquitous and tissue-specific trans-acting factors [2,3,11,12]. In contrast, artificial constructs encompassing only minimal promoter and enhancer elements [e.g. a TATA box 5′-flanked with either two glucocorticoid response elements (GRE/PRE, where PRE stands for progesterone response element) or the vitellogenin A2 oestrogen response element (ERE)] have allowed the demonstration that steroid hormone receptors can form functional pre-initiation complexes with RNA polymerase II and other transcription activating factors associated with the basal transcriptional machinery [13–19].

Only a few studies on transcription in vitro have made use of hormone-inducible genomic promoter sequences as DNA templates for the study of steroid hormone receptor-dependent transcription [20–22], and in no case were such promoters able to drive tissue-specific transcription. Even the Xenopus laevis vitellogenin B1 promoter, which is exclusively transcribed in the liver of female X. laevis toads in vivo, is unable to drive liver-specific transcription in vitro on its own. It is as active in the presence of crude nuclear extracts prepared from an established cell line of X. laevis kidney (B3.2) and from human HeLa cells as in the presence of those prepared from female X. laevis livers [23].

Abbreviations used: AdML, adenovirus major late promoter; CNEs, crude nuclear extracts; ds-oligo, double-stranded oligonucleotide; EMSA, electrophoretic mobility-shift assay; ERE, oestrogen response element; GR, glucocorticoid receptor; GRE, glucocorticoid response element; hGRα, recombinant human glucocorticoid receptor; HPICE, high-performance ion-exchange chromatography; PRE, progesterone response element; SI9-NEs, nuclear extracts prepared from SI9 insect cells; TA, tranilicine acetamide; TAT, tyrosine aminotransferase; TATα35, 5′ flanking sequences of the TAT gene spanning nt −350 to +1 (TAT promoter).

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implying that additional mechanisms are instrumental in the repression of gene expression in non-hepatic cells.

We have previously shown that the TAT promoter (TAT\textsubscript{\(n=23\)} spanning nt \(-350\) to \(+1\)) is able to drive liver-specific transcription in vitro [2,3]. Here we report that a single GRE/PRE (the TAT GREII), even when located at a remote 5’-position (\(-350\) bp relative to the transcription’s initiation site), is able to enhance transcription from TAT\textsubscript{\(n=23\)} in the presence of rat liver crude nuclear extracts, provided that these nuclear extracts were supplemented with partly purified recombinant human glucocorticoid receptor (hGR\textsubscript{\(z\)}).

**MATERIALS AND METHODS**

Plasmons and recombinant baculovirus

pAdML\textsubscript{\(n=23\)} (where AdML refers to adenovirus major late promoter), pAdML\textsubscript{\(n=23\)} FTAT\textsubscript{\(n=23\)} and pRS'hGR\textsubscript{\(z\)} have been described previously by Sawadogo and Roeder [24], Vaulont et al. [12], Schweizer-Groyer et al. [2,3] and Hollenberg et al. [25] respectively.

Construction of pGRE-TAT\textsubscript{\(148\)}

A 173 bp fragment encompassing a 148 bp ‘G-free’ cassette was amplified by PCR with p(CAT)\textsubscript{\(n=9\)} [24] as a template and the following primers: 5’-GAATTCGGTACCAGGTGCAGTCCTGA-3’; 3’-CTCTAGAAGAAGACCCTCGTTTAAATGTA-5’. The fragment was then gel-purified and restricted with SsI and BamHI (underlined restriction sites in the 5’ and 3’ primers respectively).

A chimeric TAT-GREII/TAT-promoter (GRE-TAT\textsubscript{\(n=23\)}) was also engineered by PCR amplification with pTC10 [9] as a template, and the following primers: forward 5’-TCAGAATTTCAATTACATTTGCCTTTTCCCCTATCC-3’; reverse 5’-TATGAAGCTAGCTAGTTACGAGATAGAAATGAGTG-3’. The fragment was then gel-purified and restricted with SsI and BamHI (underlined restriction sites in the 5’ and 3’ primers respectively).

Both restricted PCR fragments were mixed together and with EcoRI and BamHI-digested p\(1_{\text{T,T}}\)18U. Ligations of these fragments yielded pGRE-TAT\textsubscript{\(148\)} (see Figure 1A). Its sequence was confirmed by dye-deoxy sequencing.

Construction of baculovirus–hGR\textsubscript{\(z\)} recombinant viruses (BachGR\textsubscript{\(z\)})

hGR\textsubscript{\(z\)} cDNA sequences (insert) were excised from pRS'hGR\textsubscript{\(z\)} as a 3 kb KpnI–XhoI fragment, and vector DNA (pBlueBac; Invitrogen) [26] was linearized by restriction at the unique NheI site. After filling-in with Klenow DNA polymerase, blunt-ended vector and insert DNA species were ligated. The recombinant pBlueBac plasmids were co-transfected into Spodoptora frugiperda (Sf9) cells together with wild-type viral DNA by using the calcium phosphate co-precipitation procedure [27]. The plagues that corresponded to BachGR\textsubscript{\(z\)} were selected [they became blue in the presence of the \(\beta\)-galactosidase indicator Bluo-Gal (150 \(\mu\)g/ml)] and then purified by three rounds of plague purification.

The hGR\textsubscript{\(z\)} protein synthesized in BachGR\textsubscript{\(z\)}-infected cells was of the expected molecular mass and was able to bind glucocorticoid agonists and antagonists.

**Cell culture, transfection and infection**

COS-7 cells were maintained in exponential-phase monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10 \% (v/v) foetal calf serum/50 \(\mu\)g/ml gentamicin/2 mM l-glutamine. Transfection was performed by the DEAE-dextran procedure as previously described [28].

Sf9 cells were grown as spinner cultures in TNM-FH medium (Sigma) supplemented with 10 \% (v/v) steroid-depleted foetal calf serum (10-TNM) [29]. Sf9 cells were routinely infected with BachGr\textsubscript{\(z\)} recombinant virus at a multiplicity of more than 10. Briefly, 6 ml of 10-TNM containing approx. 6 \(\times\) 10\(^7\) cells was mixed with 3 ml of recombinant virus suspension; 2 h after infection, the cells were diluted with 60 ml of fresh 10-TNM and grown as spinner cultures for an additional 46 hours.

When indicated, 1 \(\mu\)M (final concentration) of [\(^3\)H]triamcinolone acetonide (TA) or [\(^3\)H]RU486 (RU) was added to the culture medium 1 h before cell harvest.

**Isolation of hGR\textsubscript{\(z\)} for transcription in vitro**

pRS'hGR\textsubscript{\(z\)}-transfected COS-7 cells were harvested 48 h after transfection, washed and homogenized as previously described [28]. The homogenate was centrifuged at 105000 \(\times\) g for 60 min (0–4°C), and the high-speed supernatant was fractionated by high-performance ion-exchange chromatography (HIPEC) with a Mono Q column [30]. The hGR\textsubscript{\(z\)}-containing fraction (0.32 M NaCl elute; 0.5 pmol hGR\textsubscript{\(z\)}/ml) was concentrated and desalted with Centricon 30 microconcentrators. The 0.32 M NaCl eluate obtained by Mono Q fractionation of high-speed supernatants prepared from uninfected COS-7 cells yielded preparations of mock-purified hGR\textsubscript{\(z\)}.

BachGR\textsubscript{\(z\)}-infected Sf9 cells (6 \(\times\) 10\(^7\) cells) were harvested by centrifugation and washed with 5 ml of chilled PBS; the nuclear extracts were prepared by the method of Elliston et al. [31], with minor modifications. All subsequent steps were performed at 0–4°C. The washed cell pellet was resuspended and lysed in 4 ml of hypotonic buffer (HMKD; 10 mM Hapes (pH 7.9)/15 mM MgCl\textsubscript{2}/10 mM KCl/2 mM dithiothreitol) and centrifuged at 3000 \(\times\) g for 10 min. Except when cytosoil was to be prepared, the supernatant was discarded and the pellet was centrifuged for an additional 10 min at 27000 \(\times\) g. The small volume of remaining supernatant was discarded, and the final nuclear pellet was resuspended in 1:1 immediately 5’ of the KpnI site (italics in the forward primer). After amplification this fragment was gel-purified, then restricted with EcoRI and SsI (underlined restriction sites in the 5’ and 3’ primers respectively).

Both restricted PCR fragments were mixed together and with EcoRI and BamHI-digested p\(1_{\text{T,T}}\)18U. Ligations of these fragments yielded pGRE-TAT\textsubscript{\(148\)} (see Figure 1A). Its sequence was confirmed by dye-deoxy sequencing.

**Transcription assay in vitro**

Crude liver nuclear extracts (liver CNEs) were prepared by the method of Gorski et al. [11]. The transcription assay in vitro was
performed as described in Schweizer-Groyer et al. [2,3]. The sequence of addition of reagents is shown in Figure 1(B). Each transcription assay contained two DNA templates: pTAT275, or pGRE-TAT148 (400 ng), from which transcription was driven by TAT promoter (TAT8,23) or chimaeric TAT promoter/enhancer sequences (GRE–TAT8,23), and pAdML270 or pAdML375 (100 ng), internal controls from which transcription was driven by AdML (Figure 1A). Correct initiation of transcription from these templates gave rise to transcripts of 375, 148, 280 and 385 nt respectively.

Electrophoretic mobility shift assay (EMSA)

Double-stranded poly(dI)–poly(dC) (1 μg) was mixed with 10 μg of crude liver nuclear extracts in the presence or absence of recombinant hGRα (0.4 pmol, Sf9-NEs prepared from Sf9 cells that had been infected with BachGRα) and incubated at 0 °C for 15 min. 32P-Labelled GRE/PRE (perfect palindrome; see below) (1 ng; approx. 30,000 c.p.m.) was then added either alone or in the presence of a 25–100-fold molar excess of unlabelled double-stranded oligonucleotide (ds-oligo) (GRE/PRE or ERE; see below). The final reaction mixture [20 μl; 20 mM HEPES (pH 7.4)/4 mM dithiothreitol/37.5 mM KCl/2.5 mM NaCl/l mM EDTA/75 μM MgCl2/0.5% BSA/10% (v/v) glycerol] was incubated for an additional 60 min at 25 °C, then analysed by non-denaturing PAGE [5% (w/v) gel] run in 0.25× Tris/borate/EDTA buffer at 0–4 °C. Gels were dried and DNA–protein interactions were detected by autoradiography (24–48 h at room temperature).

When supershift experiments were to be performed, the 60 min incubation at 25 °C was extended for an additional 20 min after the addition of anti-GR polyclonal antibody in the reaction mixture (1 μl) [33].

Western blot analysis

Western blotting was performed as previously described [28]. The rabbit anti-hGR polyclonal antibody was raised against a synthetic peptide (amino acid residues 149–168 of the hGR), and was able to interact with both native and SDS-treated hGR. Depending on the experiment, dexamethasone (Vectastain ABC) or chemiluminescent (ECL System; Amersham) peroxidase staining were used.

Oligonucleotides

Two ds-oligos were used as competitors, both in the transcription experiments in vitro and in EMSA: the ERE from the Xenopus laevis vitellogenin gene [34], 5’-GTCAAGTCAGGTCA CAGTGACTGATCAAAGTT-3’, and a perfectly palindromic GRE/PRE, 5’-GATCATCTGCGAGAACAGTGTTCTAGCTACTTGATC-3’.

Quantitative analyses

Authentic transcripts generated from the AdML, TAT8,23 and GRE–TAT8,23 promoters were quantified by densitometric scanning of the autoradiograms (Bio-Image System; Millipore). For each experiment, multiple exposures (in the range 8–48 h) were scanned. The amount of transcripts generated from TAT8,23 and from GRE–TAT8,23 promoters was always standardized relative to that generated from AdML, and this ratio (TAT/AdML relative transcription) was used to compute the glucocorticoid receptor (GR)-mediated enhancement of transcription: Stimulation relative to control (TAT/AdML relative transcription measured in the presence of hGRα) divided by (TAT/AdML relative transcription measured in the absence of hGRα).

RESULTS

The glucocorticoid-responsive template (pGRE-TAT148) used in the transcription experiments in vitro (Figure 1A) contained the rat TAT gene GREII sequences lying between nt −2523 and −2488 (i.e. including the CCAAT motif located between nt −2523 and −2506) [8]. This motif was included because transient transfection experiments reported by Strähle et al. [35] have shown (i) that the 15 bp GRE/PRE itself is unable to mediate hormone inducibility when inserted 351 bp upstream of the TAT gene’s cap site, and (ii) that the GR acts synergistically with the trans-acting factor (probably C/EBP) [9] that interacts with this CCAAT motif.

Figure 1 Transcription vectors and design of the transcription reaction in vitro

(A) ‘Open weave’ boxes represent full-length (375 bp: pAdML375, pTAT375) or shortened (148 bp: pGRE-TAT148; 270 bp: pAdML270) ‘G-free’ cassettes. AdML 5’ flanking sequences (nt −340 to +1) and nt +1 to +10 of the AdML transcript are depicted as hatched and filled boxes respectively. Open and checked boxes represent promoter (nt −350 to +1) and GREII (nt −2523 to −2488) sequences from the TAT gene respectively. (B) Test (pGRE-TAT148 or pTAT375; 400 ng) and 100 ng of control (pAdML375 or pAdML270) DNA templates, the transcription reaction mixture (without rNTPs), 48 μg of liver CNES and hGRαs that had previously been preincubated at 0 °C for 15 min in the presence of 0.5 μg of salmon sperm DNA were sequentially mixed as shown. After an additional 10 min of incubation at 0 °C, the transcription reaction was started, adding the rNTPs, and transcription was performed at 30 °C for 45 min.
GR–GRE/PRE complexes was ascertained both by competition experiments (it was extinguished by 25- and 100-fold molar excesses of unlabelled GRE/PRE but not by a 100-fold molar excess of ERE) (Figure 2, lanes 3–5) and by immunological recognition of hGRα (hGRα–GRE/PRE complexes were super-shifted when anti-GR polyclonal antibodies were added to the reaction mixture) (Figure 2, lane 6).

The hGRα used in the transcription experiments in vitro: origin and molecular form

Two sources of hGRα (i.e. isolated from Sf9 cells infected with BachGRα or from COS-7 cells transiently transfected with pRShGRα) were routinely used in the transcription experiments in vitro.

When BachGRα-infected Sf9 cells were cultured in 10-TNM in the absence of (anti)-hormone, no immunoreactivity against GR could be detected by Western blot analysis of the crude nuclear extracts (Figure 3, lane 1, ‘hGRα-depleted’ Sf9-NEs). Accordingly, immunoreactivity against GR was recovered at 90 kDa in the low-speed (800 g, 10 min; Figure 3, lane 5) and in the high-speed (cytosol; Figure 3, lane 4) supernatants prepared from the homogenate of these cells.

In contrast, when such an analysis was performed with BachGRα-infected Sf9 cells that had previously been cultured in 10-TNM in the presence of 1 μM steroid (TA or RU486), a strong immunoreactivity was observed in the Sf9-NEs (Figure 3, lanes 2 and 3), but was undetectable in the low-speed supernatant (Figure 3, lane 6). When such Sf9-NEs were analysed by glycerol gradient ultracentrifugation, hGRα sedimented as the transformed 3–4 S form (results not shown).

Similarly, after HPIEC fractionation of high-speed supernatants prepared from pRShGRα-transfected COS-7 cells, the partly purified hGRα was recovered as the 3–4 S transformed form (results not shown), consistent with the use of high salt conditions during the ion-exchange (Mono Q) procedure [30].

hGRα enhances transcription in vitro from pGRE-TATα8

The final design of the transcription reaction outlined in Figure 1B resulted from preliminary experiments showing that transcription in vitro was inhibited when the hGRα preparations obtained either from COS-7 cells or from Sf9 cells had not been preincubated with non-specific salmon sperm DNA before addition to the transcription mixture (G. Schweizer-Groyer, unpublished work). Moreover because the preincubation of template DNA species with CNEs is essential for the generation of ‘rapid-start’ preinitiation complexes, the formation of which is enhanced by progesterone, glucocorticoid and oestrogen receptors [13,14,18], a 10 min lag was applied before initiation of the transcription reaction (addition of rNTPs in the reaction mixture) (Figure 1B).

In most cases the basal level of transcription (i.e. constitutive transcription from the GRE-TATα8 promoter/enhancer elements) measured in the absence of hGRα-containing extracts was high when liver CNEs were used as a source of general trans-acting factors (Figure 4A, lane 1; Figure 4B, ○; Figure 5A, lanes 1, 2, 4 and 6; Figure 6, lane 1). This phenomenon could be explained by the fact that liver CNEs contain both the liver-specific and ubiquitous trans-acting factors that are required for optimal transcription from TATα8 promoter sequences [2,3].

When increasing amounts of Sf9-NEs containing TA–hGRα complexes (range 20–750 fmol of hGRα) were added to the transcription mixture, the variation in TAT/AdML relative transcription was biphasic: it increased in a dose-dependent...
Glucocorticoid receptor-enhanced *in vitro* transcription

Figure 4  hGRα expressed in Sf9 and in COS-7 cells enhances transcription *in vitro* from GRE-TAT0.35

(A) Aliquots of Sf9-NEs prepared from uninfected Sf9 cells (1.8 µl; lane 1) or from BachGRα-infected Sf9 cells (0.23–4.7 µl; lanes 2–6) were added to the transcription mixture; the reaction proceeded as outlined in Figure 1(B). The molar ratios of hGRα to pGRE-TAT148 ranged from 0.5 to 10 and are indicated above each lane (GR/template). AdML and GRE-TAT (at the right) indicate correctly initiated control and test transcripts respectively. The increase in transcription mediated by the addition of hGRα in the transcription reaction (stimulation relative to control) was computed as outlined in the Materials and methods section, and is indicated below each lane. (B) In each reaction mixture the amounts of transcript generated from GRE-TAT0.35 were standardized relative to those generated from AdML, and the corresponding ratio (TAT/AdML relative transcription) was plotted as a function of hGRα input (lower abscissa) or of GR/template (upper abscissa). (C) Aliquots (4.5–13.5 µl) of pooled, hGRα-containing HPIEC fractions were added to the transcription mixture (GR/template; range 1–3); the reaction proceeded as outlined in Figure 1(B). In each reaction mixture the amounts of transcript generated from GRE-TAT0.35 were standardized relative to those generated from AdML, and the corresponding ratio (TAT/AdML relative transcription) was plotted as a function of hGRα input (lower abscissa) or of GR/template (upper abscissa).

manner at GR-to-template ratios below 3.75 (between 30 and 250 fmol of added hGRα), was maximal up to a GR-to-template ratio of 5 (between 250 and 350 fmol of added hGRα), then decreased when this ratio was larger (more than 350 fmol of added hGRα). At optimal GR-to-template ratios (3.75–5.00), transcription from the GRE-TAT8.35 promoter was stimulated 3.2–3.5-fold relative to that obtained in the absence of added receptor (Figures 4A, lanes 2–6, and 4B).

That the presence of hGRα in the Sf9-NEs was indispensable in enhancing transcription from GRE-TAT8.35 was supported by the results shown in Figure 5(A). Transcription was not enhanced (i) when Sf9-NEs were prepared from Sf9 cells that had been infected with wild-type baculoviral DNA (i.e. it does not drive hGRα expression in Sf9 cells) (Figure 5A, lane 2) and (ii) when Sf9-NEs (1.0 or 1.6 µg of protein) that do not contain TA-hGRα complexes (i.e. prepared either from uninfected Sf9 cells, or from BachGRα-infected Sf9 cells that had not been preincubated with the agonist before homogenization; see Figure 3) were added to the transcription mixture *in vitro* (Figure 5A, lanes 1 and 6–8).

A dose-dependent stimulation of transcription (maximum 3-fold) was also obtained when partly purified TA–hGRα complexes (i.e. HPIEC fractions isolated from COS-7 cells transiently transfected with pRShGRα) were added to the transcription mixture (Figure 4C). With this source of receptor, the GR-to-template ratio for maximal enhancement of transcription was 2.5 (180 fmol of added hGRα). In contrast, transcription was not
enhanced when partly purified TA–hGRα complexes were replaced by mock-purified hGRα (i.e. HPIEC fractions isolated from untransfected COS-7 cells) (Figure 5A, compare lanes 4 and 5).

Finally, when nuclear extracts of HeLa cells were used instead of liver CNEs as a source of general trans-acting factors, transcription from GRE-TAT\textsubscript{\textasciitilde} was identical in the presence (hGRα-containing SF9-NEs) and in the absence (hGRα-depleted SF9-NEs) of hGRα in the reaction mixture (Figure 5B, compare lane 11 with lanes 9 and 10). These results support the conclusion that the presence of liver-specific trans-acting factor(s) is a prerequisite for hGRα to enhance transcription from GRE-TAT\textsubscript{\textasciitilde}.

Enhancement of transcription by hGRα is dependent on GR–GRE/PRE interaction

We next addressed the point of whether or not hGRα-mediated stimulation of transcription was dependent on hGRα-GRE/PRE interaction.

In the experiment shown in Figure 6, transcription from GRE-TAT\textsubscript{\textasciitilde} was enhanced 4.5-fold on the addition of TA–hGRα.
Glucocorticoid receptor-enhanced in vitro transcription

Antagonist (RU486)-hGR complexes stimulate transcription in vitro

Incubation of BachGRα-infected S9 cells with 1 µM RU486 yielded RU486-hGRα complexes in the nuclear extracts (see Figure 3, lane 3). Such nuclear extracts were able to enhance transcription in vitro from GRE-PRE-containing template and (ii) that this enhancement is dependent on [RU486–hGRα]–GRE/PRE interactions.

DISCUSSION

Previous results obtained in our laboratories have demonstrated that the TAT promoter (TATα35) is able to drive liver-specific transcription in vitro [2,3]. We now report that the GR is able to enhance transcription in vitro from TATα35, provided that (i) a
GRE/PRE is inserted 5' to the promoter sequences in the reporter construct and (ii) the rat liver CNEs are supplemented with recombinant glucocorticoid-receptor.

When transcription in vitro was performed in the presence of rat liver CNEs alone, the transcriptional activity of GRE-TAT\textsubscript{α,35} was identical irrespective of the presence or absence of dexamethasone in the transcription mixture. This is in contrast with what has previously been reported for oestradiol-dependent transcription with Xenopus laevis liver CNEs [20], and could be due either to the constitutive activity of already transformed GR molecules that are present in the crude liver nuclear extracts or to the lack of GR in such liver CNEs. Highly sensitive EMSA analysis of rat liver CNEs supported the latter hypothesis.

That liver CNEs were devoid of GR might be explained either by its cytoplasmic localization in the absence of hormone or by the leakage of unliganded receptor molecules out of the nuclei during the homogenization procedure under hypoxic conditions (the rats did not receive any glucocorticoid injection before being killed). Indeed, numerous immunocytochemical studies have shown that the unliganded GR, in contrast with the hGR\textsubscript{α}

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though the DNA templates contained either multiple close GRE/PRES (mouse mammary tumour virus – long terminal repeat) or tandem GRE/PRES (or ERES) inserted approx. 20 bp upstream of minimal promoter elements (TATA box) [13,14]. This high level of transcriptional enhancement is due to synergistic induction of transcription [13], a consequence of co-operative binding of progesterone or GRs to adjacent GRE/PRES [39,40]. Moreover, this functional synergism between tandem GRE/PRES is attenuated when the hormone response elements are inserted in a remote 5' position (e.g. 100 bp upstream of the cap site in the ovalbumin promoter context) [22].

In this connection it should be emphasized that the 3–4.5-fold enhancement of transcription obtained from GRE-TAT\textsubscript{α,35} in the presence of rat liver CNEs and exogenous hGR\textsubscript{α} fits the above-mentioned results. In fact GRE-TAT\textsubscript{α,35} is a chimaeric promoter/enhancer element that encompasses a single TAT-GREII (i.e. a single GRE/PRE 5' flanked by a CCAAT sequence) inserted in a remote position (350 bp upstream of the cap site) 5' to the liver-specific TAT\textsubscript{α,35} promoter. Because the trans-acting factor that interacts with the CCAAT sequence located immediately 5' of the GRE/PRE inverted palindrome in TAT-GREII (a putative C/EBP cis-element) [9] acts synergistically with the GR in transient transfection, it might also be involved in the hGR\textsubscript{α}-dependent increase in transcription in vitro. However, owing to the remote position of the TAT-GREII in GRE-TAT\textsubscript{α,35}, one can speculate that the GRE/PRE-CCAAT synergism is attenuated in the transcription assay in vitro and that hGR\textsubscript{α} yields a 3–4.5-fold rather than a 10–30-fold enhancement of transcription. Such a functional interaction (and even a synergism) between the steroid response and the binding of trans-acting factors to promoter elements has already been shown in vitro for NF1 and an NF1-like trans-acting factor in the context of ovalbumin and vitellogenin B1 promoters respectively [22,41].

We have already shown that impaired GR transformation was the major step involved in the mechanism of RU486 anti-glucocorticoid effect [42]. Thus we have previously documented that transformed RU486-GRE complexes (i) are able to interact with GRE/PRES in vitro and (ii) are as active as agonist–GR complexes to induce gene transcription when purified and added to chick oviduct nuclei [43]. Our results in transcription in vitro are consistent with these previously reported results: RU486–hGR\textsubscript{α} complexes and TA–hGR\textsubscript{α} complexes are equally active in enhancing hGR\textsubscript{α}-mediated transcription in vitro from GRE-TAT\textsubscript{α,35}. Identical results were obtained by Tsai et al. [14]. These observations suggest that when hGR\textsubscript{α} is stripped of hsp90 and of other putative ‘inhibitory’ protein(s) the conformations of agonist–apoprotein and antagonist–apoprotein complexes are closely related, so that the DNA-binding and trans-activating domain(s) are functional, at least in our transcription assay in vitro. In this connection it has been hypothesized that not only hsp90 but also hsp70 has to be stripped from the 4 S progesterone receptor for transcription to be enhanced [18].

In the liver, TAT gene transcription is controlled by a variety of hormonal stimuli (glucocorticoids, glucagon and insulin); this control is often dependent on tissue-specific gene expression (e.g. the enhancement of TAT gene transcription by glucocorticoids and cAMP is dependent on liver-specific cis-elements, HNF3 and HNF4 respectively) (reviewed in [10]). These hormonal stimuli are transduced by different pathways (nuclear receptors, adenyl cyclase-associated receptors and cAMP-dependent protein kinase, tyrosine kinase receptors) and in some instances are
antagonistic (e.g. insulin inhibits the stimulation of TAT gene transcription induced by the glucocorticoids and by cAMP). These results suggest (i) that the cross-talk between various hormonal stimuli might converge towards either identical or at least closely overlapping-end molecular targets and (ii) that liver-specific trans-acting factors might also take part in this cross-talk (e.g. the inhibitory effects of insulin on TAT gene transcription are dependent on both the cAMP response element and HNF3 cis-acting sequence) [44].

The experiments reported here clearly show that supplementation of liver CNEs with exogenous hGRz allows liver-specific, receptor-mediated enhancement of transcription in vitro from a chimaeric reporter construct that encompasses a liver-specific receptor-mediated enhancement of transcription (ARC) and La Ligue Nationale contre le Cancer.

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REFERENCES

27. Summers, M. D. and Smith, G. E. (1987) in A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedure, Bulletin no. 155, Texas Agricultural Experiment Station and Texas A and M University, College Station, TX