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

Ghislaine SCHWEIZER-GROYER* $\ddagger$, Françoise CADEPOND*, Nicole JIBARD*, Eric NEAU $\ddagger$, Ingrid SEGARD-MAUREL*, Etienne-Emile BAULIEU* and André GROUYER $\ddagger$

*Inserm U.33, Laboratory of Hormones, 80, rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cedex, France, and $\ddagger$ Inserm U.142, Hôpital St Antoine, 184, rue du Foubourg St. Antoine, 75571 Paris Cedex 12, France

The rat tyrosine aminotransferase (TAT) gene is a liver-specific and glucocorticoid-inducible gene. Previous studies have shown that the TAT promoter (TAT_{0.35}; nt –350 to +1) is able to sustain liver-specific gene expression both in transient transfection and in a transcription assay *in vitro* [Schweizer-Groyer, Groyer, Cadepond, Grange, Baulieu and Pictet (1994) Nucleic Acids Res. 22, 1583–1592]. Here we report that the basal transcriptional activity generated from TAT_{0.35} in the presence of crude liver nuclear extracts is enhanced by added human glucocorticoid receptor (hGR α), provided that TAT_{0.35} sequences were flanked (5') with a glucocorticoid responsive unit (GREII of the TAT gene, including its 5'-CCAAT flanking sequence). Two sources of hGR α were used: nuclear extracts prepared from Sf9 insect (Sf9-NEs) cells over-expressing hGR α , and hGR α from pRShGR α -transfected COS-7 cells, enriched by high-performance ion-exchange chromatography. The enhancement of tran-

scription *in vitro* (1.5–4.5-fold) was dependent on the amount of added hGR α and independent of the nature (agonist or antagonist) of the ligand. Moreover, the hGR α -mediated stimulation of transcription was (i) dependent on GRE/progesterone response element (PRE) (it was inhibited by a 25-fold excess of GRE/PRE but not by a 100-fold excess of oestrogen response element) and (ii) receptor-dependent (Sf9-NEs prepared from uninfected Sf9 cells or from Sf9 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 TAT_{0.35} 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 1_{Liver}, 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; hGR α , recombinant human glucocorticoid receptor; HPIEC, high-performance ion-exchange chromatography; PRE, progesterone response element; Sf9-NEs, nuclear extracts prepared from Sf9 insect cells; TA, triamcinolone acetone; TAT, tyrosine aminotransferase; TAT_{0.35}, 5' flanking sequences of the TAT gene spanning nt –350 to +1 (TAT promoter).

$\ddagger$ To whom correspondence should be addressed.

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_{0.35}, 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_{0.35} in the presence of rat liver crude nuclear extracts, provided that these nuclear extracts were supplemented with partly purified recombinant human glucocorticoid receptor (hGR α).

MATERIALS AND METHODS

Plasmids and recombinant baculovirus

pAdML₃₇₅ (where AdML refers to adenovirus major late promoter), pAdML₂₇₀, pTAT₃₇₅ and pRShGR α 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-TAT₁₄₈

A 173 bp fragment encompassing a 148 bp 'G-free' cassette was amplified by PCR with p(C₂AT)₁₉ [24] as a template and the following primers: 5', 5'-AATTCGAGCTCATATATTC-TCCTTCCATACCCCTATCATCC-3'; 3', 5'-CTCTAGAGGATCCCCGGGAGTGGAATGAGAAATGAGTG-3'. The fragment was then gel-purified and restricted by *Sst*I and *Bam*HI (underlined restriction sites in the 5' and 3' primers respectively).

A chimaeric TAT-GREII/TAT-promoter fragment (GRE-TAT_{0.35}) was also engineered by PCR amplification with pTC10 [9] as a template, and the following primers: forward, 5'-TCAAGAATTCAGGTTTACCAATCTCTGCTGTACAGGATGTTCTAGCGGTACCAGAGGACCCTCGTTTAAATGTATTT-3'; reverse, 5'-TGATGAGCTCAGGTGCAGTCCTGA-3'. This 400 bp fragment encompassed 36 bp of the TAT GREII (nt -2523 to -2488; bold characters in the forward primer) linked to the 350 bp *Kpn*I-*Sst*I fragment of the TAT promoter (nt -350 to +1) immediately 5' of the *Kpn*I site (italics in the forward primer). After amplification this fragment was gel-purified, then restricted with *Eco*RI and *Sst*I (underlined restriction sites in the 5' and 3' primers respectively).

Both restricted PCR fragments were mixed together and with *Eco*RI and *Bam*HI-digested pT₇T₃18U. Ligation of these fragments yielded pGRE-TAT₁₄₈ (see Figure 1A). Its sequence was confirmed by dideoxy sequencing.

Construction of baculovirus-hGR α recombinant viruses (BachGR α)

hGR α cDNA sequences (insert) were excised from pRShGR α as a 3 kb *Kpn*I-*Xho*I fragment, and vector DNA (pBlueBac; Invitrogen) [26] was linearized by restriction at the unique *Nhe*I 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 *Spodoptera frugiperda* (Sf9) cells together with wild-type viral DNA by using the calcium phosphate co-precipitation procedure [27]. The plaques that corresponded to BachGR α were selected [they became blue in the presence of the β -galactosidase indicator Blueo-Gal (150 μ g/ml)] and then purified by three rounds of plaque purification.

The hGR α protein synthesized in BachGR α -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 μ 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 α recombinant virus at a multiplicity of more than 10. Briefly, 6 ml of 10-TNM containing approx. 6×10^7 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 μ M (final concentration) of [³H]triamcinolone acetonide (TA) or [³H]RU486 (RU) was added to the culture medium 1 h before cell harvest.

Isolation of hGR α for transcription *in vitro*

pRShGR α -transfected COS-7 cells were harvested 48 h after transfection, washed and homogenized as previously described [28]. The homogenate was centrifuged at 105 000 *g* for 60 min (0–4 °C), and the high-speed supernatant was fractionated by high-performance ion-exchange chromatography (HPIEC) with a Mono Q column [30]. The hGR α -containing fraction (0.32 M NaCl eluate; 0.5 pmol hGR α /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 untransfected COS-7 cells yielded preparations of mock-purified hGR α .

BachGR α -infected Sf9 cells (6×10^7 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 Hepes (pH 7.9)/15 mM MgCl₂/10 mM KCl/2 mM dithiothreitol] and centrifuged at 3000 *g* for 10 min. Except when cytosol was to be prepared, the supernatant was discarded and the pellet was centrifuged for an additional 10 min at 27 000 *g*. The small volume of remaining supernatant was discarded, and the final nuclear pellet was resuspended 1:1 (v/v) in 20 mM Hepes (pH 7.9)/20% (v/v) glycerol/0.55 M KCl/1.5 mM MgCl₂/0.2 mM EDTA/2 mM dithiothreitol/2 μ g/ml leupeptin/2 μ g/ml aprotinin/1% (w/v) aprotinin/20 μ g/ml PMSF (nuclear extract buffer). The efficiency of nuclear lysis was improved by using a Dounce homogenizer (B pestle, 10 strokes), and the lysate was centrifuged at 12 000 *g* for 30 min. The final supernatant was divided into aliquots (referred to hereafter as Sf9-NEs) and stored for several months in liquid nitrogen. In each Sf9-NE, hGR α was assayed by the hydroxyapatite method [32]. Its specific activity (as expressed in pmol/mg of protein) varied slightly from one preparation to the other.

Sf9-NEs were also prepared from uninfected Sf9 cells (6×10^7 cells) and from Sf9 cells that had been infected with wild-type baculoviral DNA, and were used as control Sf9-NEs (i.e. devoid of hGR α).

When required, the high-speed supernatant (cytosol) was prepared, centrifuging the post-nuclear (3000 *g*, 10 min) supernatant at 105 000 *g* for 60 min.

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: pTAT₃₇₅ or pGRE-TAT₁₄₈ (400 ng), from which transcription was driven by TAT promoter (TAT_{0.35}) or chimaeric TAT promoter/enhancer sequences (GRE-TAT_{0.35}), 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. ³³P-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/1 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, diaminobenzidine (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'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3', and a perfectly palindromic GRE/PRE, 5'-GATCATCTCTGCAGAACAGGATGTTCT-AGCTACTTGATC-3'.

Quantitative analyses

Authentic transcripts generated from the AdML, TAT_{0.35} and GRE-TAT_{0.35} 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 TAT_{0.35} and from GRE-TAT_{0.35} 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 transcrip-

tion 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 chequered 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 hGRα 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.

Figure 2 Liver CNEs do not interact with a GRE/PRE unless they are supplemented with hGR α -containing Sf9-NEs

EMSA (see the Materials and methods section) was performed in the presence of liver CNEs (10 μ g of protein) and in the presence (lanes 2–6) or absence (lane 1) of 0.4 pmol of hGR α (Sf9-NEs prepared from Sf9 cells that had been infected with BachGR α). To identify authentic GR–GRE/PRE complexes, incubations were also performed in the presence of unlabelled ERE (100-fold molar excess, lane 5) or GRE/PRE (25-fold or 100-fold molar excesses, lanes 3 and 4), or in the presence of anti-GR polyclonal antibody (aGRab; lane 6). Abbreviations: [GR] $_2$, retarded band corresponding to GR homodimers; S^{GR}, super-shifted hGR α –GRE complexes; Ori, upper limit of the gel.

Figure 3 Localization and molecular form of hGR α in the subcellular fractions prepared from BachGR α -infected Sf9 cells: comparison between cells cultured in the presence or absence of (anti)-hormone

Suspensions of BachGR α -infected Sf9 cells were grown in TNM-FH medium supplemented with 10% (v/v) foetal calf serum (steroid-depleted), then homogenized before (lanes 1, 4 and 5) or after (lanes 2, 3 and 6) a 1 h incubation in the presence of either 1 μ M TA (lanes 2 and 6) or 1 μ M RU486 (lane 3). Sf9-NEs (lanes 1–3), low-speed supernatant (800 g, 10 min) (lanes 5 and 6) or cytosol (lane 4) were then prepared and analysed by Western blot (20 μ g of protein per slot). Lanes 1–4, diaminobenzidine immunoperoxidase staining; lanes 5 and 6, chemiluminescent immunoperoxidase staining.

Rat liver CNEs are devoid of GR

Because TAT_{0.35} behaves as a liver-specific promoter [2,3], a prerequisite for efficient transcription from pGRE-TAT₁₄₈ was the use of liver CNEs. However, preliminary experiments have shown that rat liver CNEs were unable to support glucocorticoid-dependent transcription *in vitro* (results not shown). One possible explanation for such results would be that rat liver CNEs were devoid of GR. We addressed this issue with the highly sensitive electrophoretic mobility-shift assay, with a perfectly palindromic GRE (i.e. the one that displays the highest affinity for the GR) [33].

As a matter of fact, only gel-shift experiments performed in the presence of liver CNEs supplemented with exogenous hGR α (i.e. Sf9-CNEs prepared from Sf9 cells infected with BachGR α) yielded GR–GRE/PRE complexes (Figure 2, compare lanes 1 and 2). That the retarded band ([GR] $_2$) represented genuine

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 hGR α (hGR α –GRE/PRE complexes were super-shifted when anti-GR polyclonal antibodies were added to the reaction mixture) (Figure 2, lane 6).

The hGR α used in the transcription experiments *in vitro*: origin and molecular form

Two sources of hGR α (i.e. isolated from Sf9 cells infected with BachGR α or from COS-7 cells transiently transfected with pRShGR α) were routinely used in the transcription experiments *in vitro*.

When BachGR α -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, 'hGR α -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 BachGR α -infected Sf9 cells that had previously been cultured in 10-TNM in the presence of 1 μ M 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, hGR α sedimented as the transformed 3–4 S form (results not shown).

Similarly, after HPIEC fractionation of high-speed supernatants prepared from pRShGR α -transfected COS-7 cells, the partly purified hGR α 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].

hGR α enhances transcription *in vitro* from pGRE-TAT₁₄₈

The final design of the transcription reaction outlined in Figure 1(B) resulted from preliminary experiments showing that transcription *in vitro* was inhibited when the hGR α 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-TAT_{0.35} promoter/enhancer elements) measured in the absence of hGR α -containing extracts was high when liver CNEs were used as a source of general *trans*-acting factors (Figure 4A, lane 1; Figure 4B, $\circ$; 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 TAT_{0.35} promoter sequences [2,3].

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

Figure 4 hGR α expressed in Sf9 and in COS-7 cells enhances transcription *in vitro* from GRE-TAT_{0.35}

(A) Aliquots of Sf9-NEs prepared from uninfected Sf9 cells (1.8 μ l; lane 1) or from BachGR α -infected Sf9 cells (0.23–4.7 μ l; lanes 2–6) were added to the transcription mixture; the reaction proceeded as outlined in Figure 1(B). The molar ratios of hGR α 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 hGR α 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-TAT_{0.35} were standardized relative to those generated from AdML, and the corresponding ratio (TAT/AdML relative transcription) was plotted as a function of hGR α input (lower abscissa) or of GR/template (upper abscissa). (C) Aliquots (4.5–13.5 μ l) of pooled, hGR α -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-TAT_{0.35} were standardized relative to those generated from AdML, and the corresponding ratio (TAT/AdML relative transcription) was plotted as a function of hGR α 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 hGR α), was maximal up to a GR-to-template ratio of 5 (between 250 and 350 fmol of added hGR α), then decreased when this ratio was larger (more than 350 fmol of added hGR α). At optimal GR-to-template ratios (3.75–5.00), transcription from the GRE-TAT_{0.35} 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 hGR α in the Sf9-NEs was indispensable in enhancing transcription from GRE-TAT_{0.35} 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

hGR α expression in Sf9 cells) (Figure 5A, lane 2) and (ii) when Sf9-NEs (1.0 or 1.6 μ g of protein) that do not contain TA-hGR α complexes (i.e. prepared either from uninfected Sf9 cells, or from BachGR α -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-hGR α complexes (i.e. HPIEC fractions isolated from COS-7 cells transiently transfected with pRShGR α) were added to the transcription mixture (Figure 4C). With this source of receptor, the GR-to-template ratio for maximal enhancement of transcription (maximum 3-fold) was also obtained when partly purified TA-hGR α complexes (i.e. HPIEC fractions isolated from COS-7 cells transiently transfected with pRShGR α) 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 hGR α). In contrast, transcription was not

Figure 5 Stimulation of transcription by Sf9-NEs prepared from BachGR α -infected Sf9 cells or from hGR α -containing HPIEC fractions is dependent on both the hGR α and liver nuclear proteins

(A), Sf9-NEs were prepared from uninfected (lanes 1 and 6), wild-type baculoviral DNA-infected (Bac, lane 2) or BachGR α -infected (Bac GR, lanes 3, 7 and 8) Sf9 cells before (lanes 6–8, GR depleted) or after (lanes 1–3) a 1 h incubation of the cells in the presence of 1 μ M TA. HPIEC fractionation of COS-7 cytosol was performed from untransfected (mock, lane 4) or from pRShGR α -transfected (GR, lane 5) COS-7 cells. Sf9-NEs or HPIEC fractions (1.1 μ g of protein and/or GR/template = 2.5) were added to the transcription mixture; the reaction proceeded as outlined in Figure 1(B). (B) Sf9-NEs were prepared from uninfected (lane 9), wild-type baculoviral DNA-infected (Bac, lane 10) or BachGR α -infected (Bac GR, lane 11) Sf9 cells after a 1 h incubation of the cells in the presence of 1 μ M TA. Replicate aliquots of Sf9-NEs (1.1 μ g of protein and/or GR/template = 2.5) were added to the transcription mixture; the reaction proceeded as outlined in Figure 1(B). In this series of experiments, liver CNEs were replaced by HeLa cell nuclear extracts as a source of general transcription factors. For both panels, TAT/AdML relative transcription was calculated under each experimental condition compared with the appropriate control (Sf9-NEs from uninfected Sf9 cells or mock purified GR) and is expressed as fold stimulation relative to that control as outlined in the Materials and methods section for glucocorticoid-enhanced transcription.

enhanced when partly purified TA-hGR α complexes were replaced by mock-purified hGR α (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_{0.35} was identical in the presence (hGR α -containing Sf9-NEs) and in the absence (hGR α -depleted Sf9-NEs) of hGR α 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 hGR α to enhance transcription from GRE-TAT_{0.35}.

Enhancement of transcription by hGR α is dependent on GR-GRE/PRE interaction

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

In the experiment shown in Figure 6, transcription from GRE-TAT_{0.35} was enhanced 4.5-fold on the addition of TA-hGR α

Figure 6 Stimulation of transcription by Sf9-NEs prepared from BachGR α -infected Sf9 cells is dependent on GREs

(A) Sf9-NEs were prepared from wild-type baculoviral DNA-infected (Bac, lane 5) or from BachGR α -infected (BachGR α , lanes 2–4, 6) Sf9 cells after a 1 h incubation of the cells in the presence of 1 μ M TA. Replicate aliquots of Sf9-NEs (lanes 2–4, 1.6 μ g of protein, GR/template = 3.75; lanes 5 and 6, 1.1 μ g of protein, GR/template = 2.5) or an identical volume of nuclear extract buffer (lane 1) were added to the transcription mixture; the reaction proceeded as outlined in Figure 1(B). When indicated above the lane, a ds-oligo encompassing authentic ERE (100-fold molar excess) or GRE/PRE (25-fold molar excess) was added to the reaction mixture together with the DNA template. AdML and TAT or GRE-TAT (at the right of each panel) indicate correctly initiated control and test transcripts respectively. TAT/AdML relative transcription was calculated under each experimental condition compared with the appropriate control (nuclear extract buffer or Sf9-NEs prepared from Sf9 cells infected with wild-type baculoviral DNA) and is expressed as stimulation relative to that control as outlined in the Materials and methods section for glucocorticoid-enhanced transcription.

complexes (270 fmol; 1.6 μ g of Sf9 nuclear proteins) to the transcription mixture *in vitro*. The magnitude of hGR α -mediated enhancement of transcription was decreased to 2.5-fold in the presence of competing GRE/PRE in the reaction mixture (25-fold molar excess) but remained unchanged (4.3-fold) in the presence of a 100-fold molar excess of ERE (Figure 6, lanes 1–4).

Moreover, when GRE-TAT_{0.35} was replaced by TAT_{0.35} (which does not contain GREII sequences upstream of the TAT gene's promoter sequences), transcription was identical whether Sf9-NEs had been prepared from Sf9 cells infected with wild-type baculoviral DNA or from Sf9 cells infected with BachGR α (Figure 6, compare lanes 5 and 6).

Taken together, these results support the conclusions that the stimulation of transcription obtained in the presence of nuclear extracts prepared from BachGR α -infected Sf9 cells was dependent both on the GR and on its interaction with its cognate response elements (i.e. the GREII sequences flanking the TAT_{0.35} promoter element in pGRE-TAT₁₄₈).

Antagonist (RU486)-hGR complexes stimulate transcription *in vitro*

Incubation of BachGR α -infected Sf9 cells with 1 μ M RU486 yielded RU486-hGR α complexes in the nuclear extracts (see Figure 3, lane 3). Such nuclear extracts were able to enhance transcription *in vitro* from GRE-TAT_{0.35} (2.1-fold stimulation relative to control) (Figure 7, lanes 1 and 2). RU486-hGR α -enhanced transcription was completely abolished in the presence of a 25-fold molar excess of GRE/PRE in the transcription mixture, but remained unchanged (2.8-fold stimulation) in the presence of ERE (100-fold molar excess) (Figure 7, lanes 3 and 4).

These results support the conclusions (i) that the transformed RU486-hGR α complexes present in the Sf9-NEs are able to stimulate transcription *in vitro* from a GRE/PRE-containing template and (ii) that this enhancement is dependent on [RU486-hGR α]-GRE/PRE interactions.

Figure 7 hGR α -RU486 complexes stimulate transcription *in vitro*

Sf9-NEs were prepared from uninfected (lane 1) or BachGR α -infected (lanes 2–4) Sf9 cells after a 1 h incubation of the cells in the presence of 1 μ M RU486. Replicate aliquots (0.8 μ g of protein, GR/template = 3.75) of the nuclear extracts were added to the transcription mixture; the reaction proceeded as outlined in Figure 1(B). When indicated above the lanes, a ds-oligo encompassing an authentic ERE (100-fold molar excess) or GRE/PRE (25-fold molar excess) was added to the reaction mixture together with the DNA template. AdML and GRE-TAT (at the right) indicate correctly initiated control and test transcripts respectively. The stimulation of transcription relative to control was computed as outlined in the Materials and methods section and is indicated below each lane.

DISCUSSION

Previous results obtained in our laboratories have demonstrated that the TAT promoter (TAT_{0.35}) 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 TAT_{0.35}, 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_{0.35} 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 oestrogen-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 hypoionic 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 oestrogen and progesterone receptors, was either cytoplasmic or could easily leak out of the cell's nucleus [36,37]. Such explanations also hold true for the absence of immunoreactive hGR α in Sf9-NEs prepared from Sf9 cells infected with BachGR α and homogenized without prior incubation with TA or RU486, and for its correlated presence in low- and high-speed supernatants.

When the liver CNEs were supplemented with exogenous hGR α , the transcriptional activity driven by the chimaeric GRE-TAT_{0.35} promoter-enhancer construct was enhanced over that observed in the absence of hGR α . That this enhancement was due to the exogenous hGR α could be inferred from four observations: (i) it was not observed in the presence of Sf9-NEs prepared either from uninfected Sf9 cells, from Sf9 cells infected with wild-type baculoviral DNA or from BachGR α -infected cells that had not previously been incubated with TA or RU486 (see above), (ii) its magnitude was dependent on the amount of receptor added, (iii) it was diminished to approx. one-half in the presence of a 25-fold molar excess of competing TAT-GREII ds-oligo (but not of competing ERE at a 100-fold molar excess) in the reaction mixture, and (iv) it was not obtained when TAT-GREII sequences were omitted from the DNA template (pTAT₃₇₅). The decrease in transcriptional stimulation observed at the highest GR-to-template ratio (of 10) might be due to self-squelching, a phenomenon that has already been reported to occur *in vitro* in the presence of the [A/B]-transcriptional activation function (TAF-1) of the human progesterone receptor [38].

Finally it should be emphasized that the use of GRE-TAT_{0.35} as a chimaeric promoter/enhancer element yielded a highly liver-specific enhancement of transcription *in vitro* by hGR α .

The maximal increase in transcription (i) was in the same range whether recombinant hGR α was obtained by using insect (Sf9; 3.2–3.5-fold) or mammalian (COS-7; 2.5-fold) cells and (ii) was achieved at similar GR-to-template ratios (3.75 and 2.5 in the presence of hGR α synthesized in Sf9 and COS-7 cells respectively). These observations support the conclusion that the hGR α protein that is produced in insect and mammalian cells is equally active, and suggest that the two cell systems do not differ in post-translational protein modification, at least with regard to the transcriptional efficiency of hGR α .

When a chimaeric DNA template that encompasses a single GRE/PRE located approx. 20 bp upstream of minimal promoter elements (TATA box) was incubated with nuclear extracts of HeLa cells and purified chicken progesterone receptor (cPRa), a

maximal enhancement of approx. 5-fold was obtained in transcription experiments *in vitro* [13]. Receptor-mediated increase in transcription *in vitro* was much more efficient (10–30-fold) when 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_{0.35} in the presence of rat liver CNEs and exogenous hGR α fits the above-mentioned results. In fact GRE-TAT_{0.35} 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_{0.35} 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 α -dependent increase in transcription *in vitro*. However, owing to the remote position of the TAT-GREII in GRE-TAT_{0.35}, one can speculate that the GRE/PRE-CCAAT synergism is attenuated in the transcription assay *in vitro* and thus that hGR 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-GR complexes (i) are able to interact with GRE/PREs *in vitro* and (ii) are as able 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 α complexes and TA-hGR α complexes are equally active in enhancing hGR α -mediated transcription *in vitro* from GRE-TAT_{0.35}. Identical results were obtained by Tsai et al. [14]. These observations suggest that when hGR α is stripped of hsp90 and of other putative 'inhibitory' protein(s) the conformations of agonist-aporeceptor and antagonist-aporeceptor 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 hGR α allows liver-specific, receptor-mediated enhancement of transcription *in vitro* from a chimaeric reporter construct that encompasses a liver-specific promoter and a remote (350 bp upstream of the transcription initiation site) GRE/PRE. Such an assay will help study the functional role of liver-specific *trans*-acting factors and of their post-translational modifications (i) in the fine tuning of transcription in response to hormonal stimuli (glucocorticoid or cAMP) and (ii) in the cross-talk between signal transduction pathways at the molecular level.

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