UV cross-linking/immunoprecipitation assay: glucocorticoid receptor-adrenergic receptor gene sequence interaction

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BioTechniques 35:100-111 (July 2003)

The rat β_1 -adrenergic receptor (β_1 -AR) gene contains glucocorticoid response element (GRE) half-sites at positions -2767 and -945. In electrophoretic mobility shift assay (EMSA) experiments, neither β_1 -AR GRE half-site recognized glucocorticoid receptors (GRs) obtained from baculovirus high-level expression systems or from mammalian cells. We have developed a sensitive UV cross-linking/immunoprecipitation assay, using a 524-bp fragment containing the prototypical GRE obtained from the rat tyrosine aminotransferase promoter sequence and using antibodies recognizing mammalian GR. Using this assay, we provide evidence that rat β_1 -AR gene sequences recognize mammalian GRs expressed in mouse 3T3 cells and that the site of GR interaction does not appear to specifically contain the β_1 -AR GRE half-sites. This represents one of the first reports demonstrating the utility of a UV cross-linking/immunoprecipitation assay in the detection of mammalian GR interaction with β_1 -AR sequences, is consistent with the lack of specific DNA-GR protein complexes observed in EMSA experiments using oligonucleotide probes containing the β_1 -AR GRE half-sites, and provides evidence that mammalian GR interaction occurs at complex rat β_1 -AR gene sequences.

INTRODUCTION

Glucocorticoids can serve as direct regulators of β -adrenergic receptor (β -AR) gene transcription (1–3). Treatment of DDT₁MF-2 muscle cells with glucocorticoids results in the rapid elevation of β_2 -AR mRNA and receptor levels (1). In addition, in other established cell lines, including the C6 glioma line, glucocorticoid treatment results in either the repression or induction of β_1 -AR and β_2 -AR mRNA transcription, respectively (3,4). Dexamethasone treatment of 3T3-L1 cells, which express both β_1 -ARs and β_2 -ARs, also resulted in differential regulation of β -AR subtype mRNAs (5). Furthermore, in DDT₁MF-2 cells, dexamethasone treatment can reverse agonist-induced down-regulation of β_2 -AR mRNA and receptor levels (5,6).

Consensus matches of putative glucocorticoid response elements (GREs) (5'-GGTACANNNTGTTCT-3') have

been identified within the human β_2 -AR gene at positions -248 and -1447 (6). Prototypical GREs are composed of two hexameric half-sites (5'-GGTACA-3') that form a palindrome separated by a 3-base spacer (7). Each GRE half-site interacts with one glucocorticoid receptor (GR) monomer; GR-mediated transcriptional activation is initiated by GR homodimer or heterodimer interaction with a complete GRE site. In additional studies, six potential GREs in the rat β_2 -AR gene have been identified; the use of β_2 -AR promoter-luciferase recombinants indicates that only one of these GREs, located at positions -379 to -365, is necessary for glucocorticoid-dependent stimulation of receptor gene expression (8). Two potential GRE half-sites have been identified at positions -2767 and -945 in the rat β_1 -AR gene (Reference 9 and unpublished observations); the 25-bp region between nucleotide positions -950 and -926 has

been reported to be responsive to glucocorticoid-induced transcriptional repression using β_1 -AR promoter-luciferase recombinants in C6 transfectant cells and appears to recognize rabbit reticulolysate-generated GRs (9). The β_1 -AR GRE half-sites do not appear to constitute the complete palindromic GRE sequence, typically noted as positive GREs for their up-regulation effect on gene transcription. In addition, a novel glucocorticoid regulatory unit (GRU) that conveys positive glucocorticoid responsiveness has been identified in the ovine β_1 -AR gene; this 43-bp region, extending from positions -1274 to -1232, contains a core binding motif for homeodomain proteins, an E-box binding site for the Myc/Max family proteins, and an overlapping GRE half-site (10).

In this report, we wanted to verify the interaction of mammalian-derived GR protein in electrophoretic mobility shift assays (EMSAs), using the β_1 -AR

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sequence surrounding the GRE half-site at positions -2767 and -945. The 25-bp sequence between positions -950 and -926 of the rat β_1 -AR gene, described to interact with rabbit reticulolysate-generated GRs, did not recognize GR obtained from baculovirus expression systems or from mammalian cells. Using a sensitive UV cross-linking/immunoprecipitation assay, we provide evidence that rat β_1 -AR gene sequences recognize mammalian GRs expressed in mouse 3T3 cells and that the site of GR interaction is complex and does not appear to specifically contain the β_1 -AR GRE half-sites. This represents one of the first reports to demonstrate the utility of a sensitive UV cross-linking/immunoprecipitation assay in the detection of mammalian GR interaction with β_1 -AR sequences.

MATERIALS AND METHODS

Cell Lines and Sequence Analysis Programs

The monkey kidney CV-1 (ATCC CCL70) and mouse 3T3 (ATCC CRL 6588) cell lines were cultured in DMEM, supplemented with 2.5% heat-inactivated fetal calf serum (FCS), 7.5% calf serum, 100 U/L penicillin, 1 mg/L streptomycin, and 2.5 mM L-glutamine. Automated DNA sequencing support was provided by the Molecular & Cell Biology Core Facilities at the Oregon Health and Science University. Auxillary in-laboratory sequence analyses of β_1 -AR sequences were conducted with the use of the MacVector 5.0 program (Eastman Kodak, Rochester, NY, USA).

Oligonucleotide Probes and Primers

Oligonucleotide DNA probes for EMSA were synthesized from Sigma-Genosys (The Woodlands, TX, USA). For EMSA analyses of GREs, sense oligonucleotides and reverse complement oligonucleotides were synthesized and annealed at equal molar concentrations before use. The annealed double-stranded oligonucleotides were electrophoresed in 6% nondenaturing polyacrylamide gels, eluted in a buffer consisting of 10 mM Tris-HCl, pH 8.0,

1 mM EDTA, and 0.1 mM NaCl, subjected to several cycles of freeze (-85°C) and thaw (37°C), and then purified by organic extractions and ethanol precipitation. End-labeled probes, including PCR fragments and annealed oligonucleotides, were purified using Sephadex® G-50 columns and subjected to the Wizard™ DNA Clean Up Kit (Promega, Madison, WI, USA) before use in EMSA.

GR Reagents

The GR discovery toolkit, obtained from Affinity Bioreagents (Golden, CO, USA), contains (i) recombinant human GR expressed in a baculovirus/insect cell expression system and (ii) dsDNA containing the complete palindromic GRE (half-sites are underlined) from the rat tyrosine amino transferase (TAT) sequence (5'-CTAGGCTGTACAGGA-TGTTCTGCCTAG-3'). Polyclonal rabbit anti-glucocorticoid receptor α (GR α) antibody was also obtained from Affinity Bioreagents. In addition, we have also utilized the affinity-purified rabbit polyclonal antibody against the mouse GR α (raised against a peptide corresponding to amino acids 5–20; GR M-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). For GRE mutagenesis analyses, we obtained or synthesized four double-stranded oligonucleotides: (i) consensus GRE from Santa Cruz Biotechnology containing two half-sites (underlined) (5'-AGAGGATCTGTACAGGATGT-TCTAGAT-3'); (ii) mutagenized GRE containing two ineffective half-sites with GT-to-CA substitutions in both half-sites (5'-AGAGGATCTCAACAGGATCATCTAGAT-3') [probe name: GRE (M1 and M2)]; (iii) mutagenized GRE containing intact upstream half-site and scrambled downstream half-site (5'-AGAGGATCTGTACAGGACTGCAGAGAT-3') (probe name: GRE/M1); and (iv) mutagenized GRE containing intact downstream half-site and scrambled upstream half-site (5'-AGAGGATCCTGCAGGGATGTTCTAGAT-3') (probe name: GRE/M2 probe). β_1 -AR upstream sequence containing consensus GRE half-sites at positions -945 and -2767 were synthesized as EMSA probes: (i) β_1 -AR sequence from -970 to -920 (reverse complement 3'-TCTCTCGTGTGACGACGCGT-

CTTGTCGCGGGTCTCTCCTCCG-GAAAGACTGTCT-5') and (ii) β_1 -AR sequence from -2793 to -2743 (sense complement 5'-ACTACTCAACCGCC-CCTTCTGCTTTCTGTTCTGAAAC-ACAGGCCCTCTTTA-3').

For the development of longer β_1 -AR upstream probes containing the GRE half-sites and flanking DNA, we synthesized PCR probes internally labeled with [α -³²P]dCTP. For the development of the upstream β_1 -AR probe (703-bp fragment), we synthesized primer A (5'-GCTCTAGAGCAGAG-AGCCAGGTCATT-3'; positions -2844 to -2819) and primer B (5'-GGAATTC-CTGGGATGATAAGGCC-3'; reverse complement; positions -2142 to -2164). For the development of the downstream β_1 -AR probe (543-bp fragment), we synthesized primer C (5'-GCTCTAGAGCCACCCTACCAAGA-3'; positions -1350 to -1328) and primer D (5'-GGAATCCCTGATCGCC-GAGGGA-3'; reverse complement; positions -808 to -830). Using primer pairs A–B and C–D, PCR was conducted with the following reaction components: 1.5 mM MgCl₂, 0.2 mM all deoxynucleotides except dCTP, 0.1 mM dCTP, 0.1 mCi [³²P]dCTP, 250 ng DNA template containing the rat β_1 -AR gene sequence -3221 to -1, 100 pmol each primer, and 5 U *Taq* DNA polymerase. The reaction mixture was subjected to an initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min. A final extension at 72°C for 1 min was performed, followed by soaking at 4°C.

A similar PCR approach was also utilized in the development of a longer EMSA probe containing the complete palindromic GRE from the rat *TAT* gene (5'-CTAGGCTGTACAGGATG-TTCTGCCTAG-3'; relative positions -2505 to -2491 in the rat *TAT* gene) flanked by additional DNA amplified from the endogeneous gene. A 524-bp genomic DNA fragment containing the *TAT* upstream region -2614 to -2091 was amplified by PCR using rat genomic DNA template and upstream (TAT/GRE oligonucleotide 1: 5'-AGG-AATTCCTTCTCAGTGTTCTCTATC-3'; *Eco*RI-engineered end) and downstream (TAT/GRE oligonucleotide 2: 5'-ACGGGATCCCGTCCCTCATTCT-

TCTTAATGC-3'; reverse complement; *Bam*HI-engineered end) primers. The 524-bp fragment was subcloned into the *Eco*RI and *Bam*HI restriction endonuclease sites of pGEM 3Z, and the ensuing recombinant pJ38 was verified by sequence analyses.

EMSA and UV Cross-Linking Procedures

EMSA was performed using 32 P-labeled double-stranded oligonucleotide probes or PCR products. Nuclear extracts from cells were prepared as described in Kirigiti and Machida (11). Probes were incubated with nuclear extract in EMSA binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 5% glycerol) for 25 min at room temperature (11,12). In some cases, using methods described in Kirigiti et al. (12) and for the TAT GRE probe interaction, competition reactions were conducted using nonradioactive PCR fragments (the 524-bp DNA fragment containing the TAT upstream region) or double-stranded oligonucleotides containing the consensus TAT GRE (containing two effective half-sites), mutant TAT GRE [mutagenized GRE containing two ineffective half-sites with GT-to-CA substitutions in both half-sites; probe name: GRE (M1 and M2)], or nonrelevant sequences. For competitions using the PCR-generated β_1 -AR upstream and downstream probes, we synthesized and prepared 30-bp oligonucleotide duplexes containing either the upstream GRE half-site (β_1 -AR sequence from -2784 to -2755; half-site at position -2767) and downstream GRE half-site (β_1 -AR sequence from -961 to -932; half-site at position -945). As a nonrelevant sequence competitor, we used the 29-bp double-stranded oligonucleotide GS-1, encompassing the rat β_1 -AR sequence -396 to -367 (12). In a prior publication (12), using a UV cross-linking/immunoblot procedure, we demonstrated that the rat β_1 -AR sequence -408 to -323, which contains the GS-1 sequence as a subsequence, recognized one major protein of 117 kDa and additional minor proteins of 37 and 35 kDa; there were no UV cross-linked proteins that approached the 100-kDa

size observed for GRs. Competition reactions were based using 1 $\times$, 10 $\times$, or 20 $\times$ molar excess of nonradioactive DNA competitor. DNA-protein complexes were electrophoresed on 4% polyacrylamide gels in 1 $\times$ Tris-glycine-EDTA buffer (50 mM Tris base, 380 mM glycine, 2 mM EDTA, pH 8.8), and visualized by autoradiography (at -85°C for 18–48 h) using Kodak[®] M-5 film or by phosphorimager analysis with the Bio-Rad[®] Molecular Imager system (Bio-Rad Laboratories, Hercules, CA, USA). Antibodies used for supershift reactions include the polyclonal rabbit anti-GR α antibody obtained from Affinity Bioreagents.

For use in protein size determinations, the reaction mixtures were then placed on ice and subjected to UV irradiation for 5 min in a cross-linking oven (Stratalinker[®] oven; Stratagene, La Jolla, CA, USA; 254 nm light; power output is 100 μ J/cm²; samples were placed in a 96-well plate; well area is 32 cm²; plate was placed 3 cm from power output source). Noncovalently bound deoxynucleotides were degraded by the addition of DNase I (0.1–4 U, 37°C, 15–30 min). The reaction mixtures were then added to equal volumes of 2 $\times$ loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 2% bromophenol blue, and 20% glycerol), boiled for 5 min, and subjected to electrophoresis in an 8% polyacrylamide (acrylamide:bis-acrylamide, 29:1; supplemented with SDS) gel. Gels were transferred to nitrocellulose membranes prior to autoradiography (-85°C for 30–72 h) or phosphorimager analysis with the Bio-Rad Molecular Imager system. Proteins are denatured and radioactively tagged, permitting accurate molecular weight determinations of the molecules recognized by EMSA.

Immunoprecipitation of UV Cross-Linked DNA-Protein Complexes

In some cases, following UV cross-linking of DNA-protein complexes and DNase I digestion, 1–2 μ L specific antibody were added to the reaction mixture and allowed to incubate overnight at 4°C. PBS was added to the mixture to bring the final volume to 200 μ L. Protein A slurry (20–40 μ L; 1:1 mix-

ture of phosphate-buffered saline (PBS) and protein A-Sepharose[®] 4B beads; Sigma, St. Louis, MO, USA) was then added and allowed to mix for 2 h at 4°C. The protein A beads were washed three times by gentle centrifugation in PBS and then resuspended in 6 $\times$ loading buffer before heating at 100°C for 2 min. DNA-protein complexes released from the protein A beads are denatured and loaded into SDS-polyacrylamide gels.

Electrotransfer to Nitrocellulose Membranes and Immunoblot Procedures

In some cases, following PAGE, proteins were electrotransferred onto nitrocellulose membranes (Magna NT; 0.22 μ m pore size; Micron Separation, Westborough, MA, USA). Following procedures described previously (11), blots were pre-soaked in 1 $\times$ TBST (0.05% Tween[®] 20, 20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and then blocked with 10% dried milk in 1 $\times$ TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 1 h before the addition of affinity-purified rabbit polyclonal antibody against the mouse GR α (Santa Cruz Biotechnology). The primary antibody was then incubated for 1 h at room temperature with gentle agitation, followed by successive membrane washes (4 times, 15 min/wash, room temperature). Goat anti-rabbit conjugated-horseradish peroxidase secondary antibody was diluted (1:10,000) in antibody buffer (1.5 mL FCS + 13.5 mL 1 $\times$ TBS) and overlaid onto the blot for 1 h with gentle agitation. Membranes were then washed four times with 1 $\times$ TBST and then developed using the SuperSignal[®] Western pico chemiluminescence substrate (Pierce Chemical, Rockford, IL, USA).

RESULTS AND DISCUSSION

GREs in the Rat β_1 -AR Gene

Based on other promoter systems exhibiting negative glucocorticoid responsiveness, we conjectured that mammalian GR might not interact solely with the prototypical GRE motif (5'-TGTTCT-3'), which in the β_1 -AR gene

antibody recognition of the monkey GR contained in CV-1 cells to the GR antibodies.

Thus, specific electrophoretic mobility shifts were not observed in EMSA using oligonucleotide probes containing the rat β_1 -AR GRE half-sites and either baculovirus-generated recombinant human GR or mammalian GR obtained from monkey CV-1 cells.

UV Cross-Linking/Immunoprecipitation Assay Indicates Binding of Mouse GR to the Rat TAT Fragment Containing a Palindromic GRE and to the Rat β_1 -AR Fragments Containing Upstream Promoter Sequences

We wanted to test the utility of an established cell line expressing mammalian GRs to recognize the palindromic GRE probe and β_1 -AR GRE half-site probes. The mouse 3T3 cell line has been characterized to express GRs using the Santa Cruz Biotechnology GR antibody in immunoblot analyses (as demonstrated in the company product literature) and was experimentally validated in our laboratory (Figure 3A, extreme left lanes). In spite of a great deal of published information concerning GRs, primarily using purified receptor preparations, there are limited commercially available antibody reagents recognizing GR. In addition, it has been difficult to confirm specific cell lines or tissues as being GR-deficient and to use these materials as verified negative controls for our experiments; some previously characterized cell lines (e.g., CV-1 cells) identified as GR-negative by functional criteria contain GR-immunoreactive protein identical in size to GR identified in the mouse 3T3 cell line (see below).

A 100-kDa molecule (major immunoreactive band) was visualized by immunoblot analyses using both 3T3 and CV-1 extracts (Figure 3A); this is consistent with our positive EMSA analyses using CV-1 extracts and the palindromic GRE probe (Figure 2A). Both 3T3 and CV-1 extracts were used to validate the existence of a 100-kDa molecule, immunoreactive to Santa Cruz Biotechnology GR antibodies, in both cell lines, and to help justify the

use of CV-1 extracts as a source of GR for EMSA displayed in Figure 2. In addition, a slightly larger molecular weight component (minor immunoreactive band) of reduced band intensity was visualized in the 3T3 lane (Figure 3A); faint traces of this minor immunoreactive component were also visualized in other immunoblots of CV-1 extracts (unpublished observations). To develop an EMSA/UV cross-linking assay approach for GR-probe interaction, we synthesized PCR probes internally labeled with [α - 32 P]dCTP and subjected DNA-protein complexes to UV cross-linking and DNase I digestion. Cell extracts or UV cross-linked EMSA reactions were denatured and electrophoresed in polyacrylamide gels, electrotransferred to nitrocellulose membranes, and subjected to immunoblot analysis using antibodies immunoreactive to the mouse GR (anti-GR Santa Cruz Biotechnology antibody; Figure 3A). When the internally labeled palindromic GRE probe and CV-1 or 3T3 extracts were used in the

EMSA/UV cross-linking assay and subjected to phosphorimager analyses, we identified a predominant, radioactively tagged, cross-linked band, equivalent in molecular weight to the slightly larger immunoreactive GR component visualized by direct immunoblot analyses of cell extracts (Figure 3, B and C; see asterisks; these bands are equivalent in molecular weight to the minor immunoreactive band visualized in Figure 3A). The intensity of this predominant radioactive cross-linked band increases with higher DNase I doses (1–2 U), and all other higher molecular weight complexes observed at lower DNase I doses (0.1–0.5 U) become eliminated. The high molecular weight complexes observed at the top of the gel could represent radioactively tagged cross-linked DNA, which becomes eliminated with higher doses of DNase I. The 100-kDa molecule is observed in all lanes in the immunoblot analyses (Figure 3A), including the UV cross-linked EMSA samples, and is due to the presence of nonradioactively

Figure 2. EMSA using CV-1 extracts. (A) EMSA using the consensus palindromic GRE probe and mutant GRE probe containing obliterated double half-sites (see Materials and Methods section for probe description). (B) EMSA using β_1 -AR probes containing the sequences -970 to -920 and -2773 to -2743, both subregions containing the GRE half-site 5'-TGTTCT-3'. Radioactively tagged 1-kb ladders are used for position referencing of DNA-protein complexes and are not used for the direct determination of size.

tagged unbound GR molecules present in the original EMSA reaction. The anti-GR Affinity Bioreagents antibody, while recognizing baculovirus-expressed GR, was not able to recognize GR obtained from mouse 3T3 cells (unpublished observations) and was not used in any experiments requiring the detection of mammalian GR.

To test for the specificity of interaction of the mammalian GR with the internally labeled palindromic GRE probe, we conducted competition experiments using nonradioactive TAT GRE competitors (exact 524-bp sequence as the radioactive probe) and nonradioactive consensus or mutant GRE sequence competitors (20-bp dsDNAs containing the consensus GRE or mutagenized GRE) (Figure 4). We have also included a double-stranded, nonspecific competitor control that contains rat β_1 -AR sequence -396 to -367. This sequence, called GS-1, contains AP-2 consensus elements and interacts with a major protein of 117 kDa and additional proteins of 37 and 35 kDa (12). All competitors were used in binding reactions utilizing the PCR-

generated TAT GRE probe and 3T3 extracts in the UV cross-linking/immunoblot assay. As observed in Figure 4, we have successfully competed GR interaction with the TAT GRE probe using the homologous competitor [intensity values of 0.76 and 0.51 for 1 $\times$ and 10 $\times$ competition reactions, respectively (in top left panel), compared to a normalized value of 1.0 for probe-GR interaction alone], and the specific consensus GRE competitor (intensity values of 0.68 and 0.26 for 1 $\times$ and 10 $\times$ competition reactions, respectively, compared to a normalized value of 1.0 for probe-GR interaction alone). Competition with the latter competitor indicates that GR interaction may occur with the consensus GRE sequence plus some additional flanking DNA contained in the competitor sequence.

It is important to note, however, that competitions using higher concentrations (10 $\times$ molar excess) of the consensus GRE competitor, and to a lesser degree the homologous competitor as well, do not appear to compete reproducibly with the GR-TAT GRE probe interaction, occasionally yielding ra-

dioactive band intensity levels approaching those observed in noncompetitor control reactions. At the higher concentrations of consensus GRE competitor, as is the case with all the oligonucleotide competitors used in this study, the DNase I to DNA substrate ratio becomes substantially skewed, altering the ability of the enzyme to uniformly digest the radioactive target DNA, compared to radioactive target DNAs in control reactions lacking competitor. In our experience, the amount of DNase I for a specific DNA mass must be titrated for optimal visualization of the radioactive, UV cross-linked GR band (Figure 3). We speculate that this may also be the explanation for the occasional observation of higher intensity UV cross-linked GR bands when using increasing concentrations (10 $\times$ molar excess) of homologous DNA competitor. Retitration of DNase I may not be possible because of the need to equalize DNA mass among the different competition reactions using carrier DNA and the unexpected and variable signal quenching observed using traditional DNA carriers such as salmon sperm DNA (unpublished observations); we speculate that the carrier DNA may contain GR recognition sites, or perhaps nonspecific adsorption sites, and that the addition of varied amounts of carrier DNA to equalize mass results in variable radioactive signal quenching. In addition, in the case of the consensus GRE oligonucleotide competitors, we speculate that the interaction of GR to the TAT GRE probe is stronger, presumably because of multiple DNA-protein points of contact, than to the much shorter consensus GRE oligonucleotide competitor. Hence, the GR band intensity resulting from the consensus GRE oligonucleotide competition, unlike that observed with the homologous DNA competition, is more easily affected by the band intensity distortion resulting from the skewing of the DNase I to DNA substrate ratio. We illustrate the results shown in Figure 4 to demonstrate trends in the competitions, but with the caveat that especially in the case with the oligonucleotide competi-

Figure 3. UV cross-linking/immunoblot assays using 3T3 or CV-1 extracts. (A) Immunoblot analyses of 3T3 and CV-1 extracts and UV cross-linked EMSA reactions using CV-1 extracts for binding material. UV cross-linked EMSA reactions were treated with DNase I (2, 1, 0.5, or 0.1 U) for 30 min at 37°C. Probe used in UV cross-linking assays was the internally labeled palindromic GRE sequence amplified from the rat TAT promoter sequence. The consensus palindromic GRE probe used in previous EMSAs displayed in Figures 1 and 2 is a shorter derivative of the internally labeled palindromic GRE sequence used as an EMSA probe in Figure 3. Blot was immunostained with anti-GR Santa Cruz Biotechnology antibodies. The 100-kDa band was also visualized in product literature provided by Santa Cruz Biotechnology. (B) The blot displayed in panel A was subjected to phosphorimager analyses before antibody immunostaining; thus, panels A and B constitute a single matched experiment. UV cross-linked proteins are denatured and radioactively tagged in panel B and can be directly compared to immunoreactive GR identified in the immunoblot analyses of the cell extracts in panel A. Asterisks denote bands that are equivalent in molecular weight to the minor immunoreactive band visualized in Figure 3A. (C) Phosphorimager analyses displayed in this panel are identical with analyses displayed in panel B, with the exception that 3T3 extracts were used as binding material in panel C. The immunoblot blot portion of this experiment that matches the phosphorimager analyses in panel C yielded results that were nearly identical to those displayed in panel A. Radioactive signals were imaged using the Bio-Rad Molecular Imager FX. Protein bands in panels A, B, and C were aligned with the use of Bio-Rad prestained protein standards.

tors, only the low concentration (1× molar excess) competitor amounts would yield reproducible results.

As expected, no competition was observed using the GS-1 nonspecific competitor control, which does not contain consensus GRE sequences. In the one experiment displayed in Figure 4, no decrease in the radioactive intensity of the UV cross-linked GR molecule was observed when using either the 1× or 10× concentrations of GS-1 competitor (Figure 4; compare the 1× and 10× competition lanes; intensity values of 1.0+ and 1.0+ for the 1× and 10× competition reactions, respectively, compared to normalized value of 1.0 for the probe-GR interaction alone lane). In addition, as predicted, in the one experiment displayed in Figure 4, very limited or negligible competitions were observed when using the mutagenized GRE oligonucleotide containing two ineffective half-sites (Figure 4; compare the 1× and 10× competition lanes; intensity values of 0.81 and 0.84 for the 1× and 10× competitions, respectively, compared to normalized value of 1.0 for the probe alone lane). Repeat experiments ($n = 3$) of the 1× molar competition experiments displayed in Figure 4 yielded similar results; competitions using the homologous 524-bp competitor, specific consensus GRE competitor, GS-1 nonspecific competitor, and mutagenized GRE competitor, all conducted at 1× molar competitor excess in a single experiment, provided GR band intensity values of 0.79 ± 0.06 , 0.79 ± 0.07 , 1.0+, and 1.0+, respectively, compared to normalized values of 1.0 for the probe-GR interaction alone lanes ($\pm$ values are equivalent to 1 standard deviation). For reasons mentioned above, conducting competitions using the higher concentration (10× molar excess) of competitor yielded varied results and were not quantified in these repeat experiments.

All radioactively tagged GR molecules were verified by electrotransfer of proteins from gels to blots, with subsequent immunostaining of blots using the GR antibody (Santa Cruz Biotechnology); all radioactively tagged GR molecules were immunoreactive with the GR antibody in the immunoblot analyses (Figure 4).

We refined the UV cross-linking assay by conducting direct immunoprecip-

itations of UV cross-linked DNA-protein complexes and utilized not only the internally labeled palindromic GRE probe derived from the *TAT* gene but also longer β_1 -AR upstream and downstream probes containing the GRE half-sites and additional flanking DNA. In this experiment, UV cross-linked DNA/3T3 complexes were DNase I-treated

(2–4 U) before direct immunoprecipitation with anti-GR Santa Cruz Biotechnology antibodies. A higher DNase I dose was utilized in some cases (4 U) to ensure complete digestion. The 3T3 cell extracts and UV cross-linked, nonimmunoprecipitated DNA/3T3 complexes were also included in the electrophoretic analysis. Proteins were electrophoresed

Figure 4. Competition assays using the internally labeled TAT GRE probe and 3T3 extracts in the UV cross-linking/immunoblot assay. Competitors include the 524-bp TAT GRE fragment (exact sequence as probe), nonradioactive consensus GRE sequence (20-bp double-stranded oligonucleotide containing the consensus GRE with additional flanking DNA), nonradioactive mutagenized GRE sequence [20-bp double-stranded oligonucleotide containing mutagenized GRE (two ineffective half-sites) plus additional flanking DNA], and the GS-1 nonspecific competitor sequence. Competitors were added at 1×, 10×, and/or 20× molar excess compared to the molar concentration of internally labeled TAT GRE probe. Signal intensities for each lane were quantitated using the Quantity One software program (Bio-Rad Laboratories), subtracted for background intensities in each lane, and then normalized against the band intensity for the control interaction without competitor within each individual gel. Note that GS-1 competitions and corresponding control interaction, as well as mutant GRE competitions and corresponding control interaction, were obtained from different regions of the same gel and have been electronically juxtaposed for this illustration. Phosphorimager signals are displayed in the left panels; all corresponding immunoblot analyses for each gel are displayed immediately adjacent in the right portion of the figure.

in polyacrylamide gels and subsequently electrotransferred to nitrocellulose membranes for immunoblot analysis using the anti-GR Santa Cruz Biotechnology antibodies. This additional immunoblot step was conducted to assist in the alignment of the immunoprecipitated, radioactively tagged GR (Figure 5A, right subpanel) with molecules identified in the UV cross-linked complexes and with GR identified by direct immunoblot analysis of 3T3 extracts (Figure 5A, left subpanel). We observe that the immunoprecipitations conducted with the internally labeled palindromic GRE probe and the β_1 -AR probe containing the upstream and downstream GRE half-sites recognize a GR-immunoreactive protein that co-migrates with GR identified by direct immunoblot analysis of 3T3 extracts (compare GR immunoreactive band and radioactively tagged GR band in Figure 5, A and B). Additional bands appearing in the immunoprecipitation lanes of the immunoblot analyses (Figure 5A; refer to bands in the 65–30 kDa range) are due to immunoreactivity of the secondary antibodies in the immunoblot analysis reacting to the immunoglobulin molecules used in the initial immunoprecipitations. The 60-kDa

molecule in 3T3 extracts is sporadically observed (compare Figures 3 and 5) and was also observed in the Santa Cruz Biotechnology product literature. Note that the extreme right lane of the gel displayed in the left subpanel in Figure 5A (demonstrating immunoblot analysis of immunoprecipitated UV cross-linked GR bound to the β_1 -AR [-2844, -2142] probe) was inadvertently cropped just before blot transfer but after phosphorimager analysis, resulting in a truncated lane. The corresponding phosphorimage (Figure 5A, right subpanel) demonstrates the correct width of the lane. Repeat UV cross-linking/immunoprecipitation experiments were conducted using the internally labeled TAT GRE probe and higher amounts of anti-GR Santa Cruz Biotechnology antibodies in the immunoprecipitation reactions, resulting in an enhancement in signal intensity of the radioactively tagged immunoprecipitated GR (compare experiments displayed in Figure 5, A and B, using 1 and 3 μ L anti-GR Santa Cruz Biotechnology antibodies, respectively). Using the amplified β_1 -AR probe containing the downstream GRE half-site and higher amounts of anti-GR Santa Cruz Biotechnology antibodies (3 μ L) in the immuno-

precipitation reaction, we have also readily observed signals corresponding to the radioactively tagged immunoprecipitated GR (Figure 5B).

Using 30-bp double-stranded oligonucleotides containing the upstream β_1 -AR GRE half-site (position -2767) or downstream GRE half-site (position -945); we conducted competition reactions using the corresponding PCR-generated β_1 -AR promoter fragment probes (703-bp upstream probe containing the GRE half-site at position -2767; 543-bp downstream probe containing the GRE half-site at position -945); and 3T3 extracts. Competitions using the 30-bp oligonucleotide duplexes were conducted to help identify the sites of GR interaction with the β_1 -AR sequence. Multiple repeats of this competition experiment ($n = 4$) using the 30-bp β_1 -AR oligonucleotide duplexes demonstrated negligible competition, using a $1 \times$ molar competitor excess, between the β_1 -AR promoter fragment probes and GR (phosphorimager signals were $1.0+$ for all repeat competitions, compared to normalized value of 1.0 for the probe-GR interaction alone lanes; $n = 4$ experiments; unpublished observations). As expected, when homologous competi-

Figure 5. UV cross-linking/immunoprecipitation assays. (A) UV cross-linking/immunoprecipitation assays using the internally labeled palindromic GRE sequence amplified from the rat TAT gene and the amplified β_1 -AR probe containing the upstream GRE half-site. The 3T3 extracts were used as binding material. UV cross-linking reactions were treated with DNase I (4 U) for 30 min at 37°C; aliquots of these reactions were also immunoprecipitated with anti-GR Santa Cruz Biotechnology antibody (1 μ L). Proteins from UV cross-linking reactions and immunoprecipitation reactions were electrophoresed on a single polyacrylamide gel and subsequently electrotransferred for immunoblot analysis using the anti-GR Santa Cruz Biotechnology antibodies. This immunoblot analysis was conducted to confirm electrophoretic alignment of GR identified in 3T3 extracts (see extreme left lane in right subpanel of panel A) with the radioactively tagged GR identified in the immunoprecipitation reactions. Note that all UV cross-linked proteins are denatured and can be compared with both molecular weight size markers and with immunoreactive GR identified in the immunoblot analysis of 3T3 extract. Also note in the right subpanel that low-intensity phosphorimages of the UV cross-linking lanes have been juxtaposed with high-intensity phosphorimages of the immunoprecipitation lanes. (B, left subpanel) Repeat UV cross-linking/immunoprecipitation experiment using the internally labeled TAT GRE sequence, fresh radioisotope, and increased amounts of anti-GR Santa Cruz Biotechnology antibody (3 μ L). Note the enhancement of signal intensity of the radioactively tagged immunoprecipitated GR. (B, right subpanel) UV cross-linking/immunoprecipitation experiment using the amplified β_1 -AR probe containing the downstream GRE half-site. The experiment was conducted as described in panel A, with the exception that fresh radioisotope was used and increasing amounts of anti-GR Santa Cruz Biotechnology antibody were applied in the immunoprecipitations.

tors were used (exact 703-bp upstream and exact 543-bp downstream nonradioactive competitors), successful competitions were observed (at 1× molar competitor excess: phosphorimager signals for the 703-bp upstream and 543-bp downstream homologous competitors were 0.84 ± 0.09 and 0.80 ± 0.09, respectively, compared to normalized values of 1.0 for the probe-GR interaction alone lanes; *n* = 4 experiments). In addition, when using nonspecific GS-1 competitors (1× molar competitor excess), no decrease in the radioactive intensity of the UV cross-linked GR molecule was observed; phosphorimager signals were 1.0+ for both the 703-bp upstream and 543-bp downstream β_1 -AR promoter fragment probes, compared to normalized values of 1.0 for the probe-GR interaction alone lanes; *n* = 4 experiments; unpublished observations). For all gels, immunoblots were prepared and stained using GR antibodies to identify the exact position of the UV cross-linked band that represented GR. These experiments provide evidence that rat β_1 -AR gene sequences recognize mammalian GRs expressed in mouse 3T3 cells and that the site of GR interaction is complex and does not appear to specifically contain the GRE half-sites.

These results are not surprising, in that negative GREs, or elements recognized for glucocorticoid-induced transcriptional repression, do not typically comprise GRE half-site sequences. Negative GREs are more diverse in sequence (13,14); the negative GRE defined in the rat proopiomelanocortin promoter comprises the sequence 5'-GGAAGGTCACGTCCA-3' (13). In addition, as defined by DNase footprinting analyses, GR binds to the rat vasointestinal peptide receptor promoter at sequences unique to either the prototypical GRE or other known negative GREs (14).

This manuscript describes a sensitive UV cross-linking/immunoprecipitation assay for the detection of mammalian GR interaction with β_1 -AR sequences. This assay was validated with the use of probes containing the palindromic GRE site in the rat *TAT* gene and experimentally demonstrates GR interaction with rat β_1 -AR upstream sequences. With the use of short

double-stranded oligonucleotide competitors, it appears that GR interaction with the rat β_1 -AR upstream sequences is complex and does not directly recognize the GRE half-sites. However, we cannot preclude the possibility that other protein factors may recognize the β_1 -AR GRE half-sites or surrounding sequences to form larger transcription complexes and complete the transactivation of the corresponding promoter.

ACKNOWLEDGMENTS

In addition to Y.B., P.K. contributed substantially to this manuscript. Special thanks are extended to Matthew Todd for his assistance in the development of the extended β_1 -AR GRE recombinants. We also thank Crystal Daniels for support during earlier stages of this project. I also thank Dr. Tom Shearer for his overall support of the program. We acknowledge the help of Yibing Jia and Kalama Taylor of the Oregon National Primate Research Center Molecular Biology and Cell Culture Core Facilities, respectively, for their assistance in automated DNA sequencing and cell culture propagation. P.K. is currently in the General Clinical Research Center at the Oregon Health & Science University. X.L. is now in the Department of Medical and Molecular Genetics at the Oregon Health & Science University. This research was supported by National Institutes of Health grant nos. HL 42358 and MH 63137, and by the American Heart Association (AHA) grant no. 0250017N. C.A.M. was also a former recipient of an AHA Established Investigatorship.

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Received 3 September 2002; accepted 10 April 2003.

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