Miller, D. W., Safer, P., & Miller, L. K. (1986) in *Genetic Engineering* (Setlow, J. K., & Hollander, A., Eds.) Vol. 8, pp 277-298, Plenum Publishing Corp., New York.
Padmanabha, R., & Glover, C. V. C. (1987) *J. Biol. Chem. 262*, 1829-1835.
Pinna, L. A. (1990) *Biochim. Biophys. Acta 1054*, 267-284.
Saxena, A., Padmanabha, R., & Glover, C. V. C. (1987) *Mol. Cell. Biol. 7*, 3409-3417.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834-8838.
Summers, M. D., & Smith, G. E. (1987) *Bulletin No. 1555*, Texas Agricultural Experiment Station, College Station, TX.
Takio, K., Kuenzel, E. A., Walsh, K. A., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4851-4855.
Traugh, J. A., Lin, W. J., Takada-Axelrod, F., & Tuazon, P. T. (1990) in *Advances in Second Messenger and Phosphoprotein Research. Vol. 24: The Biology and Medicine of Signal Transduction* (Nishizuka, Y., Endo, M., & Tanaka, C., Eds.) Raven Press, New York.
Tuazon, P. T., & Traugh, J. A. (1991) in *Advances in Second Messenger and Phosphoprotein Research* (Greengard, P., & Robinson, G. A., Eds.) Vol. 23, pp 124-164, Raven Press, New York.
Vik, T. A., Sweet, L. J., & Erickson, R. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2685-2689.
Zandomeni, R., Zandomeni, M. C., Shugar, D., & Weinmann, R. (1986) *J. Biol. Chem. 261*, 3414-3419.

Stability and Sequence-Specific DNA Binding of Activation-Labile Mutants of the Human Glucocorticoid Receptor†

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ABSTRACT: The stability and DNA-binding properties of activation-labile (*act*[1]) human glucocorticoid receptors (hGRs) from the glucocorticoid-resistant mutant 3R7.6TG.4 were investigated. These receptors are able to bind reversibly associating ligands with normal affinity and specificity, but become unstable during attempted activation to the DNA binding form [Harmon et al. (1984) *J. Steroid Biochem. 21*, 227-236]. Affinity labeling and immunochemical analysis demonstrated that *act*[1] receptors are not preferentially proteolyzed during attempted activation. In addition, analysis of binding to calf thymus DNA showed that after loss of ligand, *act*[1] receptors retain the ability to bind to DNA nonspecifically. A 370 bp MMTV promoter fragment containing multiple GREs and an upstream 342 bp fragment lacking GRE sequences were used to assess the binding of *act*[1] hGR to specific DNA sequences. Immunoadsorption of hGR-DNA complexes after incubation with ^{32}P-end-labeled fragments showed that both normal and *act*[1] hGR bound selectively to the GRE-containing fragment in an activation-dependent manner. Binding of both normal and *act*[1] hGRs could be blocked with a synthetic oligonucleotide containing a perfect palindromic GRE, but not with an oligonucleotide in which the GRE was replaced by an ERE. Analogous results were obtained for normal and *act*[1] hGR activated in the absence of ligand, or after incubation with the glucocorticoid antagonist RU 38486. These results suggest that sequence-specific binding of the hGR does not require the presence of bound ligand and suggest a role for the ligand in trans-activation of hormonally responsive genes.

The ability of steroid hormone receptors to modulate the expression of hormonally responsive genes is strictly dependent upon the presence of bound ligand, and activation of the steroid-receptor complex to a form capable of sequence-specific DNA binding (Evans, 1988; Beato, 1989; Carson-Jurica et al., 1990). Analysis of proteolytic receptor fragments and site-directed mutagenesis have mapped the ligand binding domain of the glucocorticoid receptor (GR)[1] to the carboxyl-terminal one-third of the steroid binding protein (Carlstedt-Duke et al., 1987; Giguere et al., 1986; Danielsen et al., 1987; Rusconi et al., 1987). Deletion of the entire GR ligand binding domain, or of an internal segment postulated to be responsible for the interaction of the GR with hsp90, results in a receptor protein with constitutive DNA binding and trans-activating activity (Danielsen et al., 1987; Godowski et al., 1987; Hollenberg et al., 1987; Pratt et al., 1988). Conversely, coupling of the GR ligand binding domain to GAL4 (Webster et al., 1988; Hollenberg & Evans, 1988) or to the adenovirus E1A protein (Picard et al., 1988; Becker et al., 1989) results in hormone-regulated expression of the activities of the chimeric proteins. Thus, the ligand binding domain appears to directly or indirectly participate in the repression of the DNA binding and trans-activating activities of the receptor.

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[1] Abbreviations: *act*[1], activation-labile; DM, dexamethasone 21-mesylate; ERE, estrogen response element; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; hsp90, 90-kDa heat shock protein; LTR, long-terminal repeat; MMTV, mouse mammary tumor virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TA, triamcinolone acetonide.

The GR ligand binding domain also contains nuclear localization (Picard & Yamamoto, 1987) and intrinsic trans-activating activities (Hollenberg & Evans, 1988; Webster et al., 1988). In addition, a portion of this domain is highly homologous to a region of the estrogen receptor necessary for homodimer formation (Fawell et al., 1990). Thus, the ligand binding domain of the GR contains multiple and distinct activities. However, the mechanism by which the binding of ligand modulates some or all of these remains poorly understood. Although a core 16-kDa tryptic fragment which binds ligand with high affinity has been identified in the rat GR (Simons et al., 1989), mutations throughout the entire domain reduce or abolish its ligand binding activity (Danielsen et al., 1986; Giguere et al., 1986), and affinity labeling has shown that methionine-622, cysteine-656, and cysteine-754 of the rat GR are in close proximity to bound ligand (Simons et al., 1987; Carlstedt-Duke et al., 1988). Thus, numerous regions of the ligand binding domain can either influence the binding of or interact with the steroid molecule.

One role of the ligand is to promote GR activation, presumably by facilitating the dissociation of hsp90 from the heteromeric, unactivated, steroid–receptor complex, thereby exposing the nuclear localization and DNA binding activities of the protein (Sanchez et al., 1987; Denis et al., 1988). This model is supported by experiments in which treatments that promoted the activation and/or nuclear localization of agonist–receptor complexes failed to have the same effect on unliganded receptors, or on antagonist–receptor complexes (Groyer et al., 1987; Sanchez et al., 1987; Denis et al., 1988; Lefebvre et al., 1988; Raaka et al., 1989; Segnitz & Gehring, 1990). However, other laboratories have reported that under appropriate conditions both unoccupied receptors and antagonist–receptor complexes can be successfully activated to the DNA binding forms in vitro and/or undergo nuclear translocation in vivo (Willmann & Beato, 1986; Schmidt, 1986, 1989; Nemoto et al., 1990; Qi et al., 1990). Since neither unoccupied receptors nor antagonist–receptor complexes are biologically active, these results suggest a potential role for the ligand in events subsequent to receptor activation.

We have previously described a novel class of somatic mutations in the human GR (hGR) of the leukemic cell line CEM-C7 which bind steroids with normal affinity and specificity, but which when subjected to conditions which would result in the activation of normal steroid–hGR complexes rapidly lose their ability to retain bound ligand (Schmidt et al., 1980; Harmon et al., 1984a). These receptors were therefore classified as *act*[1] to reflect this unusual, conditional instability (Schmidt et al., 1980). We have now taken advantage of this novel phenotype to explore the role of the ligand in sequence-specific binding of the hGR. Our results indicate that *act*[1] receptor protein is not inherently unstable and that it shows the same preference for DNA sequences containing the GRE as does normal GR.

Experimental Procedures

Cells and Cell Growth. The isolation, characterization, and growth of the glucocorticoid-sensitive cell line 6TG1.1 and the *act*[1] cell line 3R7.6TG.4 as well as the human B-cell line IM-9 have been previously described (Harmon & Thompson, 1981; Harmon et al., 1984b, 1985).

Preparation of hGR. Cells were washed twice with 10 mL of Hanks' balanced salt solution, and the final cell pellet was frozen in dry ice. After being thawed at 0–4 °C, the pellet was extracted with 10 mM HEPES (pH 8.0), containing 1 mM EDTA, and 10% glycerol at a final concentration of 2.5 × 10^8 cell equiv/mL and centrifuged at 18000*g* for 10 min to obtain a crude cytoplasmic fraction. For labeling with reversibly associating ligands, extracts were incubated with 100 nM [^{3}H]TA (20 Ci/mmol; Amersham) for 2 h in the presence or absence of 10 μM unlabeled TA. Bound radioligand was determined by liquid scintillation counting after adsorption of unbound ligand to dextran-coated charcoal. For affinity labeling, receptor extracts were incubated with 200 nM [^{3}H]DM (37–46 Ci/mmol; New England Nuclear) (Simons & Thompson, 1981) for 3 h in the presence or absence of unlabeled dexamethasone as previously described (Smith & Harmon, 1985). Alternatively, for affinity labeling of hGR in intact cells, cells were suspended at a concentration of 2 × 10^7 cells/mL in RPMI 1640 medium containing 25 mM HEPES and incubated with 84 nM [^{3}H]DM for 3 h at 0–4 °C in the absence or presence of 4 μM dexamethasone as previously described (Harmon et al., 1989). Extracts were then prepared as described above.

Receptor Activation. Receptor activation was performed essentially as previously described (Smith et al., 1986). Labeled extracts were diluted with an equal volume of HEG buffer (10 mM HEPES, 1 mM EDTA, and 10% glycerol, pH 7.6) containing 0.1 M NaCl and activated at 23 °C for 30–45 min. Sodium molybdate was then added to both unactivated and activated samples to a final concentration of 10 mM.

DNA–Cellulose Chromatography. Samples (1.0 mL unactivated; 2.0 mL activated) containing equal amounts of cell extract (25–30 mg of protein/mL) were chromatographed on 1.0-mL columns of DNA–cellulose equilibrated in HEG buffer containing 10 mM sodium molybdate as previously described (Smith et al., 1986), with the exception that bound receptor was eluted with HEG buffer containing 30 mM $MgCl_2$.

Analysis of hGR Protein. SDS–PAGE was performed as described by Laemmli (1970) in 8% polyacrylamide gels with a 3% polyacrylamide stacking gel. In all experiments, samples representing equivalent numbers of cells were analyzed. Proteins were visualized by fluorography, or immunoblotting with anti-hGR antiserum AC40 and ^{125}I-protein A as previously described (Eisen et al., 1988). When used for immunoblotting of crude cell extracts, antiserum AC40 reacts with a number of nonreceptor proteins (cf. Figures 1–3). However, the 92-kDa component identified with this antiserum has previously been shown to represent the intact steroid binding protein of the hGR (Eisen et al., 1988).

Fragment Selection. The 370 bp *Eco*RI/*Hin*dIII fragment of plasmid pM50 (Cordingley et al., 1987) containing the sequence −223 to +107 of the MMTV promoter (strain C3H; Donehower et al., 1981) and the 342 bp *Eco*RI/*Hin*dIII fragment of plasmid pC118 containing the upstream sequence −652 to −362 inserted into pGEM3 were purified by preparative agarose gel electrophoresis and end-labeled with [α-^{32}P]dATP using the Klenow fragment of *Escherichia coli* Pol I (Maniatis et al., 1982). The 370 bp *Eco*RI/*Hin*dIII fragment derived from pM50 contains four GR binding sites, while the 342 bp upstream fragment from pC118 contains no such elements (Scheidereit et al., 1983). Labeled fragment (50–200 pmol) was added to 110 μL of receptor extract containing 5 μg of poly(dI-dC) and incubated for 30 min at 0–4 °C. In general, receptor extracts contained between 0.15 and 0.35 pmol of hGR as measured by specific [^{3}H]TA binding. Receptor–DNA complexes were adsorbed to protein A–Sepharose-immobilized anti-hGR antibodies (antiserum 884; Harmon et al., 1984b) and washed 3 times with HEG buffer containing 50 mM NaCl, and bound DNA was eluted at 65 °C for 15 min with HEG buffer containing 0.1% SDS. The

Table I: Binding of Normal and *act*^{1} hGR to DNA–Cellulose[a]

receptors	unactivated			activated		
	dpm applied	dpm bound	%	dpm applied	dpm bound	%
wild type	95078	2650	3	74300	30875	42
act^1	105835	2683	3	84068	10225	12

[a] Extracts prepared from dex^s 6TG1.1 ("wild-type") and *act*^{1} 3R7.6TG.4 (act^1) cells were labeled with [^{3}H]TA and subjected to DNA-cellulose chromatography either before or after activation as described under Experimental Procedures. All values have been corrected for nonspecific binding and represent the average of three experiments.

eluted fragments were ethanol-precipitated in the presence of 300 mM sodium acetate and 20 μg of glycogen, resuspended in 50 μL of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, and resolved by gel electrophoresis in 5% polyacrylamide gels. Labeled fragments were analyzed by autoradiography. In those experiments where receptors were labeled with agonist, [^{3}H]TA was used to monitor the efficiency of immunoadsorption; the efficiency of receptor immunoadsorption as judged by recovery of [^{3}H]TA was approximately 50%.

Oligonucleotide Competition. The synthetic oligonucleotides (35-mers) 5′-GATCCAAAGTCAGAACACAGTGTTCT-GATCAAAGA-3′ and 5′-GATCCAAAGTCAGGTCAC-AGTGACCTGATCAAAGA-3′, and their reverse complements, containing a single perfect palindromic glucocorticoid response element (GRE) and estrogen response element (ERE), respectively (Klein-Hitpass et al., 1986; Klock et al., 1987; Archer et al., 1990), were synthesized as individual strands on an ABI Model 430A oligonucleotide synthesizer. Complementary strands were mixed in 10 mM potassium phosphate buffer (pH 7.6) containing 0.1 M KCl, heated to 85 °C for 15 min, and annealed by slow cooling. For competition experiments, various amounts of double-stranded oligonucleotide were added in combination with a constant amount of labeled fragment and samples processed as above.

Results

Stability of Normal and act^1 *Receptors.* We have previously shown that when *act*^{1} receptors are subjected to conditions which would result in the activation of normal hGR, there is rapid loss of ligand from the mutant receptors (Schmidt et al., 1980; Harmon et al., 1984a). However, in these studies, it was not possible to ascertain the fate of the *act*^{1} receptor protein once the radioligand had dissociated. To determine whether *act*^{1} receptors were degraded after attempted activation, or remained intact, receptors were examined by affinity labeling and immunoblotting. Cell extracts prepared from normal and *act*^{1} cells were affinity-labeled with [^{3}H]DM and subjected to conditions which were previously shown to activate [^{3}H]DM-labeled receptors (Smith et al., 1986). Samples were fractionated by SDS–PAGE, and labeled receptor was visualized by fluorography. The results showed that in vitro activation resulted in no appreciable degradation of either normal or *act*^{1} receptors (Figure 1). The apparent lack of GR degradation seen with [^{3}H]DM-labeled GR could in part be due to the presence of the covalent affinity ligand bound to the protein. Therefore, extracts were incubated with the reversibly associating ligand TA and receptors visualized by immunoblotting. Despite the fact that in addition to its reaction with the 92-kDa hGR protein the anti-hGR antiserum AC40 reacts with a number of nonreceptor proteins (Eisen et al., 1988), the results showed that after in vitro activation there was no selective loss of 92-kDa *act*^{1} receptors (Figure 1, lanes 9–12). Identical results were obtained after activation of *act*^{1} hGR in intact cells (data not shown). Thus, *act*^{1} receptors are not more susceptible to proteolytic degradation

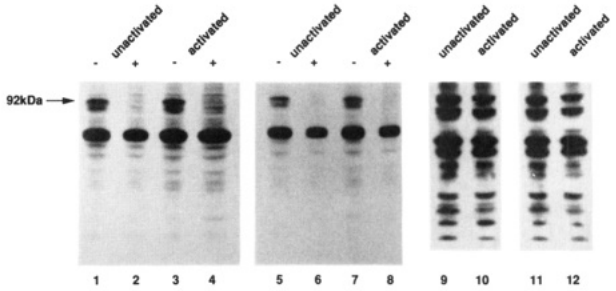

FIGURE 1: Stability of *act*^{1} receptors after attempted activation. Extracts prepared from dex^s 6TG1.1 (lanes 1–4) and *act*^{1} 3R7.6TG.4 cells (lanes 5–8) were labeled with [^{3}H]DM in the absence (odd-numbered lanes) or presence (even-numbered lanes) of an excess of unlabeled dexamethasone and fractionated by SDS–PAGE either prior to (lanes 1, 2, 5, and 6) or after (lanes 3, 4, 7, and 8) activation. hGR was visualized by fluorography. Alternatively, extracts prepared from dex^s (lanes 9 and 10) and *act*^{1} (lanes 11 and 12) cells were labeled with [^{3}H]triamcinolone acetonide and fractionated by SDS–PAGE before (lanes 9 and 11) or after (lanes 10 and 12) activation, and the hGR was visualized by immunoblotting.

than are normal receptors, indicating that the loss of ligand previously seen after attempted activation (Harmon et al., 1984a) is the result of ligand dissociation rather than receptor degradation.

Binding of act^1 *Receptor to Calf Thymus DNA.* In previous studies, activation of normal and *act*^{1} receptors was assessed by DEAE-cellulose chromatography (Schmidt et al., 1980; Harmon et al., 1984a). To examine the DNA binding properties of *act*^{1} receptors, cell extracts were labeled with [^{3}H]TA and subjected to DNA–cellulose chromatography either before or after activation. The results showed that while almost half of the ligand associated with normal receptors was retained, only a small portion of [^{3}H]TA associated with *act*^{1} receptors prior to their activation was recovered (Table I). However, when the same experiment was performed with [^{3}H]DM-labeled extracts, and the bound protein eluted and fractionated by SDS–PAGE, it was found that a significant amount of [^{3}H]DM-labeled *act*^{1} receptor was retained on the column (Figure 2). This result could represent the intrinsic ability of the *act*^{1} protein to bind to DNA–cellulose. Alternatively, it could reflect stabilization of the DNA binding activity of the *act*^{1} protein by the attachment of the covalent affinity ligand.

To differentiate these possibilities, extracts were labeled with [^{3}H]TA prior to activation and DNA–cellulose chromatography. Bound protein was eluted and fractionated by SDS–PAGE and the GR visualized by immunoblotting. The results showed that nearly as much *act*^{1} receptor protein as normal protein was retained (Figure 3). Thus, despite the fact that *act*^{1} receptors do not retain reversibly associating ligands after activation (Harmon et al., 1984a), the *act*^{1} receptor protein exhibits significant DNA binding activity, indicating that the presence of bound ligand is not required for *act*^{1} GR binding to DNA–cellulose. Indeed, when either normal or *act*^{1} receptors were subjected to conditions which activate agonist–receptor complexes, it was found that the binding of both *act*^{1}

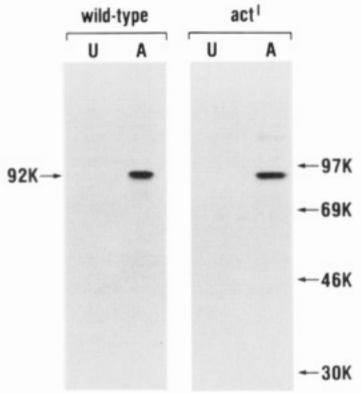

FIGURE 2: Binding of [^{3}H]DM-labeled normal and *act*^{1} hGR to calf thymus DNA. Extracts of dex^s 6TG1.1 ("wild-type") and *act*^{1} 3R7.6TG.4 (act^1) cells labeled with [^{3}H]DM were applied to columns of DNA–cellulose either before (U) or after (A) activation as described under Experimental Procedures. Labeled hGR was eluted with HEG buffer containing 30 mM $MgCl_2$, fractionated by SDS–PAGE, and visualized by fluorography. Arrows to the right indicate the positions of the [^{14}C]methylated protein standards phosphorylase *b* (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa). The arrow to the left indicates the position of the 92-kDa hGR.

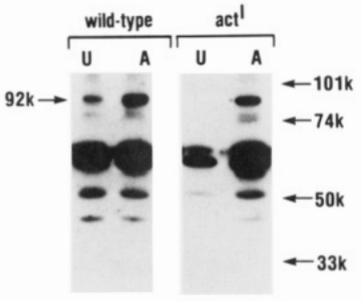

FIGURE 3: Binding of hGR protein to calf thymus DNA. Extracts prepared from dex^s 6TG1.1 ("wild-type") and *act*^{1} 3R7.6TG.4 (act^1) cells were incubated with [^{3}H]TA for 2 h at 4 °C and subjected to DNA–cellulose chromatography either before (U) or after (A) activation as in Figure 2. hGR protein was eluted with HEG buffer containing 30 mM $MgCl_2$, fractionated by SDS–PAGE, and visualized by immunoblotting as described under Experimental Procedures. Arrows to the right indicate the positions of the prestained protein standards phosphorylase *b* ($M_r \simeq 101\,000$), BSA ($M_r \simeq 74\,000$), ovalbumin ($M_r \simeq 50\,000$), and carbonic anhydrase ($M_r \simeq 33\,000$).

and normal receptors was stimulated (data not shown). Thus, at least in crude preparations of human lymphoid cell GR, bound ligand is unnecessary for nonspecific binding of either normal or mutant hGR to DNA.

Sequence-Specific Binding of act^1 *Receptors.* The experiments described above demonstrate that *act*^{1} receptors can be activated to a form which will readily bind nonspecific DNA sequences. To examine the sequence-specific binding of *act*^{1} receptors, a 370 bp *Eco*RI/*Hin*dIII fragment of plasmid pM50 containing the −223 to +107 promoter fragment of the MMTV LTR (Cordingley et al., 1987) was employed. Because of the low concentration of GR (less than 10 000 sites per cell in the *act*^{1} mutant; Harmon et al., 1984a), and because the inability of *act*^{1} receptor to retain ligand after activation precludes purification by traditional methods, we chose to immunoadsorb DNA–receptor complexes with anti-receptor antibodies rather than employ a mobility shift assay. In addition, for these experiments the anti-hGR antiserum 884 was used since we have previously shown that after immunoadsorption with this antiserum the cross-reacting proteins present in Figures 1–3 are virtually eliminated (Eisen et al., 1988). Accordingly, extracts were incubated with ^{32}P-end-labeled fragment either before or after activation, and receptor–DNA complexes were

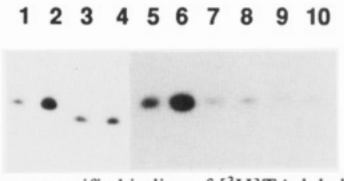

FIGURE 4: Sequence-specific binding of [^{3}H]TA-labeled IM-9 hGR. [^{3}H]TA-labeled IM-9 cell extracts were incubated with a ^{32}P-end-labeled 370 bp promoter fragment (−223 to +107) of the MMTV LTR containing 4 GREs (Scheidereit et al., 1983) (lanes 1, 2, and 5–10) or a 342 bp upstream fragment lacking any GRE sequences (lanes 3 and 4), either before (odd-numbered lanes) or after (even-numbered lanes) activation. Receptor–DNA complexes were immunoadsorbed to protein A–Sepharose-immobilized immune (lanes 1–6, 9, and 10) or nonimmune (lanes 7 and 8) anti-hGR antibodies, and bound fragment was eluted and resolved by PAGE as described under Experimental Procedures. Lanes 9 and 10 are identical to lanes 5 and 6 except that extraction buffer was used instead of receptor extracts.

immunopurified with protein A–Sepharose-immobilized anti-hGR antiserum 884. Adsorbed fragments were eluted and fractionated by polyacrylamide gel electrophoresis. The results of one such experiment, performed with IM-9 cell extract, are shown in Figure 4. A small amount of labeled fragment is bound when unactivated TA–receptor complexes are incubated with either the specific (GRE-containing) fragment (lane 1) or a nonspecific 342 bp fragment derived from an upstream region of the MMTV LTR (lane 3). This probably represents trapping of free fragment to the resin, since a comparable band is present even when nonimmune antibodies are employed (lanes 7 and 8), or when extraction buffer is substituted for receptor extract (lanes 9 and 10). However, after activation, binding was markedly enhanced to the GRE-containing fragment (lane 2) but not to the control fragment lacking the GRE sequence (lane 4). These results show that the assay employed is capable of differentiating specific and nonspecific DNA sequences and demonstrate that sequence-specific binding is dependent on receptor activation.

When the binding of wild-type and *act*^{1} CEM cell receptors was compared, it was found that after activation there was enhanced binding of both normal and mutant receptors to the GRE-containing fragment, but not to the control fragment lacking the GRE sequence (Figure 5, panels A and B), suggesting that sequence-specific binding of the *act*^{1} receptor does not require the presence of bound ligand. To more carefully compare the specificities of the normal and *act*^{1} receptor–DNA interactions, synthetic oligonucleotides containing either a perfect palindromic GRE sequence or a perfect palindromic ERE sequence were used to compete for receptor binding to the labeled GRE-containing fragment. When a 5-fold molar excess of competing GRE was employed, no competition was observed (Figure 6). However, a 25-fold excess effectively competed for the binding of both normal and *act*^{1} receptors. In contrast, no competition was seen with the ERE oligonucleotide, even at a 25-fold molar excess (Figure 6). These results show that there are no obvious differences between the sequence preferences of activated normal and *act*^{1} receptors and are therefore consistent with the interpretation that the presence of bound ligand is not required for sequence-specific binding of the hGR.

Alternatively, the lack of ligand dependence for *act*^{1} receptor binding to DNA could be a pleiotypic consequence of the *act*^{1} mutation. To explore this question, unliganded normal and *act*^{1} extracts were incubated with end-labeled fragments before or after activation. The results indicated that as had been seen for GR binding to DNA–cellulose, there was enhanced binding of both normal and *act*^{1} receptors to the GRE-containing

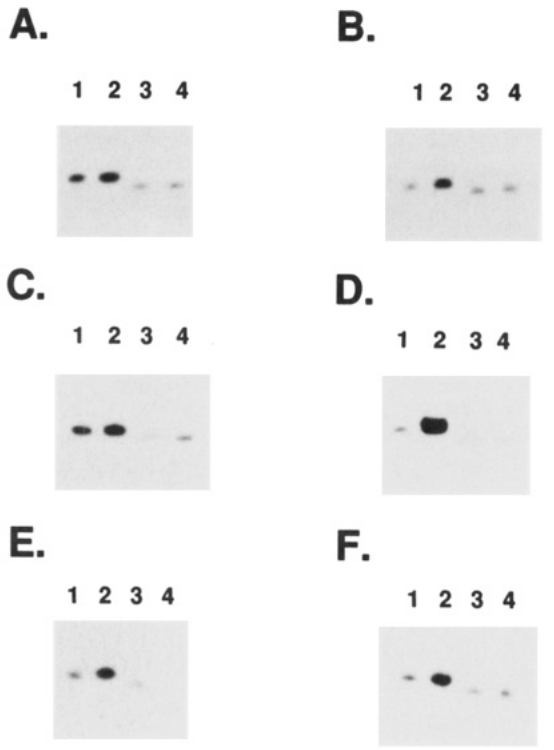

FIGURE 5: Sequence-specific binding of [^{3}H]TA-labeled normal and *act*^{1} hGR. Extracts prepared from normal (panels A, C, and E) or *act*^{1} (panels B, D, and F) cells were incubated with TA (panels A and B), no ligand (panels C and D), or RU 38486 (panels E and F) for 2 h at 0–4 °C. Labeled extracts were then incubated with the GRE-containing (lanes 1 and 2) or control (lanes 3 and 4) ^{32}P-end-labeled fragments, either before (odd-numbered lanes) or after (even-numbered lanes) activation as in Figure 4. Immunoadsorbed fragments were eluted and resolved by PAGE as described under Experimental Procedures.

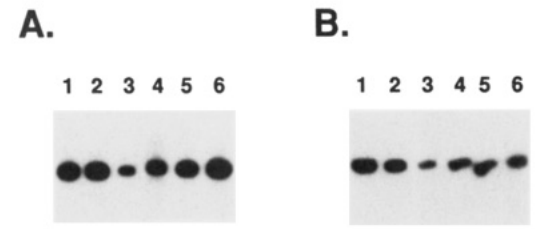

FIGURE 6: Oligonucleotide competition for sequence-specific DNA binding of normal and *act*^{1} hGR. Normal dex^s (panel A) or *act*^{1} (panel B) [^{3}H]TA-labeled extracts were incubated with the ^{32}P-end-labeled GRE-containing MMTV fragment in the presence of a 0-fold (lanes 1 and 4), 5-fold (lanes 2 and 5), or 25-fold (lanes 3 and 6) molar excess of a synthetic 35-mer containing a perfect palindromic GRE (lanes 1–3) or a perfect palindromic ERE (lanes 4–6) after activation as described under Experimental Procedures. After immunoadsorption, retained fragments were eluted and resolved by PAGE as described under Experimental Procedures.

fragment (Figure 5, panels C and D). Thus, although activation is required to express the DNA binding activity of the GR, it is clear that the presence of ligand is not obligatory for the elaboration of this activity. Indeed, even when receptors were incubated with the glucocorticoid antagonist RU 38486, there was enhanced sequence-specific binding of both normal and *act*^{1} receptors after activation (Figure 5, panels E and F). Thus, to the extent that the methods employed are capable of identifying differences in the DNA binding properties of various forms of normal and *act*^{1} hGR, there appear to be no obvious differences in the binding of normal and *act*^{1} receptors whether they are unoccupied or occupied by agonist or antagonist.

Discussion

To gain additional insight into the potential role of the ligand in modulating the DNA binding properties of the hGR, the binding properties of a novel *act*^{1} mutant of the hGR to the MMTV LTR were examined. Previous studies addressing this question have generally compared the DNA binding properties of activated agonist–receptor complexes with those of the unliganded receptor subjected to conditions which activate agonist–receptor complexes (Sanchez et al., 1987; Denis et al., 1988). Such studies are difficult to interpret because they cannot easily discriminate between inefficient "activation" of the unliganded receptor and the inability of the unliganded receptor to bind to DNA. In addition, they presume that activation of the unliganded receptor is functionally equivalent to activation of the agonist–receptor complex. Such a presumption may not be valid, since we have shown efficient in vitro activation of agonist–receptor complexes involves more than simple dissociation of the heteromeric unactivated complex (Harmon et al., 1988).

The use of *act*^{1} receptors circumvents both of these potential complications. We have previously shown that the *act*^{1} phenotype is the result of an inability of activated agonist–receptor complexes to retain previously bound ligand (Schmidt et al., 1980; Harmon et al., 1984a). In addition, in contrast to the lack of hGR nuclear translocation in some *act*^{1} clones (Harmon et al., 1984a; Antakly et al., 1990), nuclear hGR protein can be identified in clone 3R7.6TG.4 by both radioligand binding (Harmon et al., 1984a) and immunochemical detection.[2] Thus, activation of 3R7.6TG.4 *act*^{1} receptors more closely mimics activation of normal agonist–receptor complexes than does attempted activation of unliganded receptors. As expected, normal hGR occupied with agonist showed a distinct, activation-dependent, preference for an MMTV fragment containing multiple GREs. *act*^{1} receptors showed the same preference, demonstrating that they can readily differentiate sequences containing and lacking the GRE. More importantly, binding of both normal and *act*^{1} receptors to the GRE-containing fragment was effectively competed with an excess of an oligonucleotide containing a perfect palindromic GRE, but not with an oligonucleotide containing a perfect ERE, and which differed in sequence at only 4 of 35 positions. The extents of competition observed at 5 and 25 molar excesses of competing GRE oligonucleotide were similar, indicating the affinities of normal and *act*^{1} receptors for the MMTV LTR are not distinctly different. These results suggest that the ligand does not play a crucial role in modulating the actual interaction of the receptor with DNA and are consistent with the observation that C-terminal GR truncations lacking the ligand binding domain retain their sequence-specific DNA binding properties in vitro (Rusconi & Yamamoto, 1987). They are also consistent with the observation that both C-terminal truncations and internal deletions, presumably lacking the region responsible for interaction of the receptor with hsp90, and which do not bind hormone, are constitutive (albeit with reduced efficiency) trans-activators when transfected into cells lacking endogenous receptor (Danielsen et al., 1987; Godowski et al., 1987; Hollenberg et al., 1987; Pratt et al., 1988).

The results obtained with unliganded receptors or receptors occupied with RU 38486 were comparable to those obtained for *act*^{1} GR activated in the presence of agonist. Although several laboratories have reported that there is little "activation" of the GR in the absence of agonist or in the

[2] Elsasser and Harmon, unpublished results.

presence of antagonist (Groyer et al., 1987; Sanchez et al., 1987; Denis et al., 1988; Lefebvre et al., 1988; Raaka et al., 1989; Segnitz & Gehring, 1990), no such difficulty was encountered with hGR from CEM cells. Indeed, in CEM cells, despite significant nuclear accumulation of antagonist–hGR complexes (Schmidt, 1989), RU 38486 is a full antagonist (Schmidt, 1986). Most probably this indicates that there is a relatively low concentration of endogenous inhibitors of activation in CEM cells (Meshinchi et al., 1990).

The results presented here suggest that the ligand does not play a major role in determining the specificity of GR binding to DNA. However, there is significant nuclear accumulation of hGR in some *act*^{1} mutants, (Harmon et al., 1984a), and it is therefore difficult to explain why these mutants do not respond to hormone if the role of the ligand is simply to promote receptor activation. One possibility is that in vitro binding to DNA is not an accurate reflection of the binding of GR to chromatin in vivo. Alternatively, the ligand may be involved in steps distal to DNA binding. Several lines of evidence suggest that this is the case. Webster et al. (1988) showed that protein chimeras of the yeast transcription factor GAL4 DNA binding domain and the steroid binding domain of the glucocorticoid receptor bind to GAL4-responsive elements when occupied with either agonist or antagonist. However, only agonist was capable of stimulating transcription. In addition, when a C-terminally truncated, constitutively active, progesterone receptor and an intact, steroid-dependent progesterone receptor were cotransfected into cells, addition of RU 38486 resulted in repression of a hormonally responsive reporter gene (Guiochon-Mantel et al., 1988). Most directly, while it has been reported that stimulation of in vitro transcription by GR or estrogen receptors expressed in insect cells is hormone-independent (Elliston et al., 1990; Tsai et al., 1990), stimulation of in vitro transcription by estrogen or progesterone receptors from other sources shows a strict requirement for bound agonist (Corthesy et al., 1988; Kalff et al., 1990; Bagchi et al., 1990), as does GR trans-activation in yeast (Picard et al., 1990). Thus, the inability of *act*^{1} receptors to mediate a biological response may be a direct consequence of their inability to retain bound ligand necessary for transcriptional activation.

It is also possible that the methods employed here were not sensitive enough to detect subtle differences between the DNA binding properties of normal and *act*^{1} receptors. Schauer et al. (1989) found that despite the fact that the liganded and unliganded forms of the rat liver GR produced virtually identical DNase I protection patterns on the MMTV LTR, the affinity of the unliganded GR for the GRE was greater than the affinity of agonist–GR complexes. If this is the case for *act*^{1} receptors, then it might be expected that such receptors would function as dominant repressors when coexpressed with normal receptor. However, in somatic hybrids of normal and *act*^{1} cells, there was no evidence for the presence of such repressor activity (Harmon et al., 1985).

Finally, the conformation of the receptor–DNA complex may vary depending on whether agonist is present. Such a situation has been described for the progesterone receptor (El-Ashry et al., 1989) and could influence the ability of the receptor to interact with other components of the transcription apparatus in vivo. Differentiation of these possibilities will require the cloning and expression of the *act*^{1} receptor to allow more sophisticated analyses than those described here.

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References

Antakly, T., O'Donnell, D., & Thompson, E. B. (1990) *Cancer Res. 50*, 1337–1345.

Archer, T. K., Hager, G. L., & Omichinski, J. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7560–7564.

Bagchi, M. K., Tsai, S. Y., Tsai, M.-J., & O'Malley, B. W. (1990) *Nature (London) 345*, 547–550.

Beato, M. (1989) *Cell 56*, 335–344.

Becker, D. M., Hollenberg, S. M., & Ricciardi, R. P. (1989) *Mol. Cell. Biol. 9*, 3878–3887.

Carlstedt-Duke, J., Strömstedt, P.-E., Wrange, Ö., Bergman, T., Gustafsson, J.-Å., & Jörnvall, H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4437–4440.

Carlstedt-Duke, J., Strömstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-Å., & Jörnvall, H. (1988) *J. Biol. Chem. 263*, 6842–6846.

Carson-Jurica, M. A., Schrader, W. T., & O'Malley, B. W. (1990) *Endocr. Rev. 11*, 201–220.

Cordingley, M. G., Riegel, A. T., & Hager, G. L. (1987) *Cell 48*, 261–270.

Corthésy, B., Hipskind, R., Theulaz, I., & Wahli, W. (1988) *Science 239*, 1137–1139.

Danielsen, M., Northrop, J. P., & Ringold, G. M. (1986) *EMBO J. 5*, 2513–2522.

Danielsen, M., Northrop, J. P., Jonklaas, J., & Ringold, G. M. (1987) *Mol. Endocrinol. 1*, 816–822.

Denis, M., Poellinger, L., Wikström, A.-C., & Gustafsson, J.-Å. (1988) *Nature (London) 333*, 686–688.

Donehower, L. A., Huang, A. L., & Hager, G. L. (1981) *J. Virol. 37*, 226–238.

Eisen, L. P., Elsasser, M. S., & Harmon, J. M. (1988) *J. Biol. Chem. 263*, 12044–12048.

El-Ashry, D., Oñate, S. A., Nordeen, S. K., & Edwards, D. P. (1989) *Mol. Endocrinol. 3*, 1545–1558.

Elliston, J. F., Fawell, S. E., Klein-Hitpass, L., Tsai, S. Y., Tsai, M.-J., Parker, M. G., & O'Malley, B. W. (1990) *Mol. Cell. Biol. 10*, 6607–6612.

Evans, R. M. (1988) *Science 240*, 889–895.

Fawell, S. E., Lees, J. A., White, R., & Parker, M. G. (1990) *Cell 60*, 953–962.

Giguère, V., Hollenberg, S. M., Rosenfeld, M. G., & Evans, R. M. (1986) *Cell 46*, 645–652.

Godowski, P. J., Rusconi, S., Miesfeld, R., & Yamamoto, K. R. (1987) *Nature (London) 325*, 365–368.

Groyer, A., Schweizer-Groyer, G., Cadepond, F., Mariller, M., & Baulieu, E.-E. (1987) *Nature (London) 328*, 624–626.

Guichon-Mantel, A., Loosfelt, H., Ragot, T., Bailly, A., Atger, M., Misrahi, M., Perricaudet, M., & Milgrom, E. (1988) *Nature (London) 336*, 695–698.

Harmon, J. M., & Thompson, E. B. (1981) *Mol. Cell. Biol. 1*, 512–521.

Harmon, J. M., Schmidt, T. J., & Thompson, E. B. (1984a) *J. Steroid Biochem. 21*, 227–236.

Harmon, J. M., Eisen, H. J., Brower, S. T., Simons, S. S., Jr., Langley, C. L., & Thompson, E. B. (1984b) *Cancer Res. 44*, 4540–4547.

Harmon, J. M., Thompson, E. B., & Baione, K. A. (1985) *Cancer Res. 45*, 1587–1593.

Harmon, J. M., Elsasser, M. E., Urda, L. A., & Eisen, L. P. (1988) *J. Steroid Biochem. 31*, 275–281.

Harmon, J. M., Elsasser, M. S., Eisen, L. P., Urda, L. A., Ashraf, J., & Thompson, E. B. (1989) *Mol. Endocrinol. 3*, 734–743.

Hollenberg, S. M., & Evans, R. M. (1988) *Cell 55*, 899–906.
Hollenberg, S. M., Giguere, V., Segui, P., & Evans, R. M. (1987) *Cell 49*, 39–46.
Kalff, M., Gross, B., & Beato, M. (1990) *Nature (London) 344*, 360–362.
Klein-Hitpass, L., Schorpp, M., Wagner, U., & Ryffel, G. U. (1986) *Cell 46*, 1053–1061.
Klock, G., Strähle, U., & Schütz, G. (1987) *Nature (London) 329*, 734–736.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lefebvre, P., Danze, P.-M., Sablonniere, B., Richard, C., Formstecher, P., & Dautrevaux, M. (1988) *Biochemistry 27*, 9186–9194.
Maniatis, T., Fritch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Meshinchi, S., Sanchez, E. R., Martell, K. J., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 4863–4870.
Nemoto, T., Mason, G. G. F., Wilhelmsson, A., Cuthill, S., Hapgood, J., Gustafsson, J.-Å., & Poellinger, L. (1990) *J. Biol. Chem. 265*, 2269–2277.
Picard, D., & Yamamoto, K. R. (1987) *EMBO J. 6*, 3333–3340.
Picard, D., Salser, S. J., & Yamamoto, K. R. (1988) *Cell 54*, 1073–1080.
Picard, D., Khursheed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S., & Yamamoto, K. R. (1990) *Nature (London) 348*, 166–168.
Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer-Groyer, G., Catelli, M.-G., Evans, R. M., & Baulieu, E.-E. (1988) *J. Biol. Chem. 263*, 267–273.
Qi, M., Stasenko, L. J., & DeFranco, D. B. (1990) *Mol. Endocrinol. 4*, 455–464.
Raaka, B. M., Finnerty, M., & Samuels, H. H. (1989) *Mol. Endocrinol. 3*, 332–341.
Rusconi, S., & Yamamoto, K. R. (1987) *EMBO J. 6*, 1309–1315.
Sanchez, E. R., Meschinchi, S., Tienrungroj, W., Schlesinger, M. J., Toft, D. O., & Pratt, W. B. (1987) *J. Biol. Chem. 262*, 6986–6991.
Schauer, M. S., Chalepakis, G., Willmann, T., & Beato, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1123–1127.
Scheidereit, C., Geisse, S., Westphal, H. M., & Beato, M. (1983) *Nature (London) 304*, 749–752.
Schmidt, T. J. (1986) *J. Steroid Biochem. 24*, 853–863.
Schmidt, T. J. (1989) *Cancer Res. 49*, 4390–4395.
Schmidt, T. J., Harmon, J. M., & Thompson, E. B. (1980) *Nature (London) 286*, 507–510.
Segnitz, B., & Gehring, U. (1990) *J. Biol. Chem. 265*, 2789–2796.
Simons, S. S., Jr., & Thompson, E. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3541–3545.
Simons, S. S., Jr., Pumphrey, J. G., Rudikoff, S., & Eisen, H. J. (1987) *J. Biol. Chem. 262*, 9676–9680.
Simons, S. S., Jr., Sistare, F. D., & Chakraborti, P. (1989) *J. Biol. Chem. 264*, 14493–14497.
Smith, A. C., & Harmon, J. M. (1985) *Biochemistry 24*, 4946–4951.
Smith, A. C., Elsasser, M. S., & Harmon, J. M. (1986) *J. Biol. Chem. 261*, 13285–13292.
Tsai, S. Y., Srinivasan, G., Allan, G. F., Thompson, E. B., O'Malley, B. W., & Tsai, M.-J. (1990) *J. Biol. Chem. 265*, 17055–17061.
Webster, N. J. G., Green, S., Jin, J. R., & Chambon, P. (1988) *Cell 54*, 199–207.
Willmann, T., & Beato, M. (1986) *Nature (London) 324*, 688–691.