Characterization and Sequence-Specific Binding to Mouse Mammary Tumor Virus DNA of Purified Activated Human Glucocorticoid Receptor†

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ABSTRACT: Activated glucocorticoid receptor (GR) from the human cell line HeLa S_3 was purified by differential chromatography on DNA–cellulose followed by DEAE-Sepharose chromatography to 50–60% homogeneity according to sodium dodecyl sulfate gel electrophoresis and densitometric scanning of silver-stained gels. These gels routinely demonstrated a main band of M_r 94 000 (94K band) and two minor bands of M_r 79 000 (79K band) and 39 000 (39K band), respectively. Photoaffinity labeling indicated that the hormone was bound to the 94K and 79K components. In some preparations, a 72K band was observed. Further characterization of the purified receptor by gel permeation chromatography on Sephadex G-200 revealed a receptor complex with a Stokes radius of 5.8 nm. The sedimentation coefficient of the purified receptor was 4.4 S_w. In analogy to the rat hepatic GR, limited proteolysis of the purified GR with trypsin or α-chymotrypsin led to degradation of the 94K and 79K components and appearance of 28K and 39K fragments, respectively. In addition, no difference in the protease digestion pattern using *Staphylococcus aureus* V8 protease was observed. Immunoblotting using a monoclonal antibody raised against the 94K GR from rat liver demonstrated cross-reactivity with the human 94K and 79K proteins from HeLa S_3 cells, indicating similar antigenic characteristics between rat and human GR. In our study, five out of nine tested monoclonal antibodies against the rat liver GR cross-reacted with human GR. DNase I and exonuclease III protection experiments demonstrated binding of the purified human GR to specific GR binding regions in mouse mammary tumor virus DNA. Both the purified rat and human receptors recognized the same nucleotide sequences in mouse mammary tumor virus DNA exhibited a similar degree of DNase I and exonuclease III protection at similar concentrations of receptor. The similarity in structure and specific DNA binding suggests highly conserved properties of GR from different species.

Glucocorticoids affect the growth and differentiated functions of virtually every animal tissue (Rousseau & Baxter, 1979). These effects are mediated through the glucocorticoid receptor (GR),[1] an intracellular soluble protein which binds glucocorticoid hormones with high affinity and specificity (Grody et al., 1982). The GR from rat liver has been purified and extensively characterized (Wrange et al., 1979, 1984; Govindan, 1979; Eisen & Glinsmann, 1978). The role of GR in the biological action of glucocorticoid hormones is strengthened by the findings that glucocorticoid resistance is correlated to changes in the concentration and/or physical properties of GR in primary cortisol resistance in human (Chrousos et al., 1982) and in various mutant cell lines such as S49, P1798, and hepatoma cells (Stevens et al., 1983).

Experimental data indicate that the purified rat GR (rGR) recognizes specific DNA sequences in vitro which are relevant for glucocorticoid sensitivity in vivo and which are located near or within regulated genes (Yamamoto, 1985). In contrast to the rGR, there exists limited data concerning the molecular properties and DNA binding characteristics of the hGR. Recently, cDNA cloning of both the hGR and the rGR has indicated a strong homology in the deduced primary structure of both proteins (Hollenberg et al., 1985; Miesfeld et al., 1986). On the other hand, studies of the hGR in crude cellular extracts have suggested differences in molecular properties between the rGR and the hGR (Hoschützky & Pongs, 1985). In the present study, we have attempted to develop a purification protocol for the hGR from HeLa cells in order to compare structural and functional properties of the purified hGR and rGR proteins.

Experimental Procedures

Materials. Glassware was siliconized with silicone solution (Serva, Heidelberg, West Germany). SDS, acrylamide, *N*,*N*′-methylenebis(acrylamide), and reagents for immunoblotting (described below) were purchased from Bio-Rad (Richmond, CA). DTT, α-chymotrypsin (75 units/mg), and trypsin (140 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Sources of other materials have been given previously (Wrange et al., 1979). [6,7-^{3}H]TA (specific radioactivity 1.10–1.85 TBq/mmol) was purchased from Amersham (Amersham Corp., Buckinghamshire, England) and

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[1] Abbreviations: hGR, human glucocorticoid receptor; rGR, rat glucocorticoid receptor; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; TA, triamcinolone acetonide [9α-fluoro-11β,21-dihydroxy-16α,17α-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3,20-dione]; dexamethasone (dex), 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; estradiol, 1,3,5(10)-estratriene-3,17β-diol; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; GAM–HRP, horseradish peroxidase labeled goat anti-mouse immunoglobulin; TBS, 20 mM Tris and 500 mM NaCl, pH 7.5; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

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routinely diluted to a specific radioactivity of 300 kBq/mmol with unlabeled TA. [1,2,4,6,7-^{3}H]Dexamethasone (specific activity 2.92 TBq/mmol; Amersham Corp.) was diluted to a specific radioactivity of 800 kBq/mmol with unlabeled dexamethasone.

Preparation and Labeling of Cytosol. HeLa S_3 cells were grown in suspension culture in Dulbecco's modified essential medium (Gibco, Ltd., England) supplemented with 5% (v/v) fetal calf serum, L-glutamine (2 mM), benzylpenicillin (400 IU/mL), and streptomycin (0.2 mg/mL) and harvested at a cell density of (1.0–2.0) × 10^6 cells/mL. After homogenization of the cells in buffer A [1 mM Na_2EDTA, 20 mM sodium phosphate, pH 7.0, 10% (w/v) glycerol, 10 mM DTT, and 50 mM NaCl], using an all-glass Dounce B homogenizer and 20 manual strokes, cytosol was prepared by centrifugation at 100000g_{av} for 70 min at 4 °C. Cytosol was labeled as described by Wrange et al. (1979).

Protein concentrations in cytosols were determined according to Lowry et al. (1951). Binding capacity (receptor sites per cell or femtomoles of receptor per milligram of protein) and dissociation constants (K_D) were calculated according to Scatchard (1949). The effect of various ligands on the specific binding of 1 × 10^{-9} M [^{3}H]TA to cytosol from HeLa S_3 cells was determined by incubation 0.5-mL aliquots of cytosol in the absence or presence of increasing concentrations of unlabeled dexamethasone, progesterone, corticosterone, 5α-dihydrotestosterone, or estradiol. Specific binding was calculated by subtraction of the radioactivity bound in the presence of a 200-fold molar excess of unlabeled TA. Free TA and bound [^{3}H]TA were separated by treatment with dextran-coated charcoal as described above. Preparation of cytosol and labeling of receptor from lymphocytes were carried out as described for HeLa S_3 cells.

Purification of rGR. Hepatic rGR was purified as described by Wrange et al. (1984). Briefly, the activated[2] GR was purified by sequential chromatography on DNA–cellulose and DEAE-Sepharose and analyzed by sodium dodecyl sulfate gel electrophoresis, demonstrating a main band with M_r 94 000 and minor bands with M_r 79 000 and 72 000.

Purification of hGR. Human GR was purified by differential chromatography on phosphocellulose and DNA–cellulose followed by DEAE-Sepharose chromatography essentially as described for the hepatic rGR (cf. above). To optimize the procedure for smaller receptor quantities, the volumes of phosphocellulose and DNA–cellulose were reduced to 30 and 15 mL, respectively. Furthermore, the original purification procedure (Wrange et al., 1979) has been subject to minor modifications, the most important being the exclusion of the first DNA–cellulose column. In this study, the hGR has been purified according to the modified protocol (Wrange et al., 1986).

Approximately (2–4) × 10^{11} cells were used for each purification. The cytosolic fraction was incubated with 100 nM [^{3}H]triamcinolone acetonide at 0 °C for 30 min and then rapidly passed through a 30-mL phosphocellulose column (Whatman P11; 2 × 4 cm; flow rate 50–80 mL cm^{-2} h^{-1}) equilibrated in buffer A. The yellow-white flow-through fraction was diluted with an equal volume of buffer A and incubated at 25 °C for 30 min in order to activate the steroid receptor complex (Wrange et al., 1979). After activation, the diluted flow-through was chromatographed on a 15-mL DNA–cellulose (Alberts & Herrick, 1971) column (3.0 × 2.6 cm; flow rate 10 mL cm^{-2} h^{-1}) equilibrated in buffer A. The column was washed with (i) 30 mL of buffer B [1 mM Na_2EDTA, 20 mM Tris-HCl, pH 7.8, 10% (w/v) glycerol, and 10 mM DTT] containing 90 mM NaCl, (ii) 20 mL of buffer B containing 115 mM NaCl, and (iii) 20 mL of buffer B without NaCl. GR was eluted with 30 mL of buffer B containing 25 mM $MgCl_2$ and finally washed with 30 mL of buffer B containing 0.3 M NaCl (Wrange et al., 1984). The $MgCl_2$-eluted material was pooled (usually 10 mL) and applied on a 2-mL DEAE-Sepharose column (1.0 × 1.4 cm; flow rate 20 mL cm^{-2} h^{-1}). This column was eluted with a linear gradient of 0–0.6 M NaCl in 50 mL of buffer B. The GR peak was eluted at 0.12 M NaCl, as determined by conductivity measurement. Aliquots of the purified receptor (0.5–2.0 μg at 1–3 μg/mL) were stored at −70 °C in 20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 20% (w/v) glycerol, 120 mM NaCl, 20 mM DTT, 250 μg/mL insulin, and 1 μM TA.

Gel permeation chromatography was carried out by using a 75 × 2.6 cm column of Sephadex G-200 equilibrated in buffer B, containing 115 mM NaCl, 0.02% (w/v) sodium azide, and 100 μg of insulin/mL at a flow rate of 8 mL cm^{-2} h^{-1}. The column was calibrated with the following standard proteins: horse spleen ferritin (6.1 nm), methylated ^{14}C-labeled bovine serum albumin (3.6 nm), and methylated ^{14}C-labeled ovalbumin (2.7 nm). Prior to use for GR experiments, the Sephadex G-200 column was coated with 10 mg of human serum albumin/mL to avoid unspecific adsorption of the purified GR to the column.

Glycerol Density Gradient Centrifugation. hGR (0.2 pmol) in a final volume of 200 μL of buffer A was applied on a 12–50% (w/v) 4.4-mL glycerol gradient in buffer A containing 150 mM NaCl and 100 μg of insulin/mL. Parallel gradients contained ^{14}C-labeled catalase (11.3 S) and ^{14}C-labeled ovalbumin (3.5 S). Gradients were centrifuged for 17 h at 350000g at 0–2 °C, and fractions were collected in 200-μL aliquots from the bottom. Each fraction was assayed for radioactivity.

SDS–Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in slab gels (separation gel 160 × 140 × 0.75 mm) of 12% (w/v) acrylamide in a discontinuous buffer system (Laemmli, 1970) as previously described (Wrange et al., 1984). The molecular weight calibration kit used (Pharmacia, Uppsala, Sweden) consisted of phosphorylase *b* (M_r 97 400), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 000), and α-lactalbumin (M_r 14 400).

Proteolytic Digestion of GR. Two micrograms of DEAE-Sepharose-purified GR in buffer B, pH 7.8, containing 100 μg of insulin/mL, was digested with 0.8 μg of α-chymotrypsin and trypsin, respectively. Each sample was kept at 10 °C for 30 min and then cooled on ice and trichloroacetic acid precipitated. One microgram of hGR in buffer B, pH 7.8, containing 100 μg of insulin/mL, was also digested with 0.1 μg of *Staphylococcus aureus* V8 protease at 20 °C for 5 and 15 min, respectively. The GR preparations were then subjected to electrophoresis followed by silver staining of the gel as described above.

GR Quantitation. The amount of human GR was calculated by assuming M_r 94 000 and one hormone-binding site per GR molecule as for the rat liver GR (Wrange et al., 1979).

GR Photoaffinity Labeling. The purified [^{3}H]TA-labeled activated hGR was labeled as described by Wrange et al. (1984).

[2] After binding of hormone ligands, steroid receptor proteins undergo a process referred to as either transformation or activation, resulting in a conversion of the protein from a non-DNA binding form to a species that binds to DNA in vitro or nuclei in vivo. This process will in the following be referred to as activation.

Radioactivity was measured in a 1216 Rackbeta II liquid scintillation counter (LKB-Wallac, Stockholm, Sweden) as previously described (Wrange et al., 1979).

Second-Antibody Immunoprecipitation Assay. [^{3}H]TA–hGR or [^{3}H]dex–hGR complexes from HeLa S_3 cell and lymphocyte cytosols (0.2 pmol) were incubated with 50 μL of ascites containing monoclonal antibodies and 40 μL of normal mouse serum in a final volume of 0.2 mL of buffer A containing 0.15 M NaCl for 2 h at 4 °C. GR–antibody complex was precipitated by adding 50 μL of undiluted rabbit anti-mouse immunoglobulin (Dako Immunoglobulins). After incubation for 50 min at 4 °C, precipitates were pelleted by centrifugation and washed twice with 1 mL of buffer A containing 0.15 M NaCl and 100 μg of insulin/mL. Following dissolution of the pellets in 1 M NaOH and neutralization with 1 M HCl, radioactivity was measured. Nonspecific binding was determined in tubes containing [^{3}H]TA- and [^{3}H]dex–hGR complexes and medium from Sp 2/0 cells or normal mouse serum. Samples were scored positive if precipitated radioactivity was 2–3 times the amount of nonspecific binding.

Immunoblotting. After electrophoresis (cf. above), gel slabs were applied to nitrocellulose paper in an electrophoretic transfer apparatus (Transblot, Bio-Rad). The tank was filled with buffer C [20 mM Tris-HCl, 150 mM glycine, and 20% methanol (v/v)] and electrophoresis carried out at 180 mA for 12 h. The immunostaining of hGR was performed according to an available protocol (Bio-Rad Laboratories) using a horseradish peroxidase labeled goat anti-mouse immunoglobulin (GAM-HRP)(Bio-Rad Laboratories). Briefly, the nitrocellulose filters were incubated with 3% (w/v) gelatin in 20 mM Tris and 500 mM NaCl, pH 7.5 (TBS), followed by an incubation with the indicated monoclonal antibody against rGR in ascites, diluted to 1:500 in TBS with 1% (w/v) gelatin. After being washed with TBS, the GAM–HRP was applied. After a final washing, the insoluble substrate 4α-chloronaphthol was used to visualize protein recognized by the monoclonal antibody. No color reaction occurred if Sp 2/0 ascites were substituted for the primary antibody.

DNase I and Exonuclease III Footprinting. Plasmid pLS5′139 contains the GR-binding sites 1.3 and 1.4 and the upstream half of 1.5 constructed by 5′ deletions of binding region 1 of mouse mammary tumor virus (Wrange et al., 1986). For DNase I protection experiments, a 185 bp *Sst*I–*Eco*RI fragment of pLS5′ was end labeled with [α-^{32}P]dATP (9.25 MBq/mmol, Amersham, England) using the *Escherichia coli* large (Klenow) fragment of DNA polymerase I (BRL, Gaithersburg, MA). For exonuclease III protection experiments, the same fragment was 5′ labeled by T4 polynucleotide kinase (Boehringer Mannheim, FRG) and [γ-^{32}P]dATP (9.25 MBq/mmol, Amersham, England). In each reaction, approximately 1–2 nM labeled DNA was incubated in the presence or absence of purified rat or human receptor (0–20 nM) in 100–400-μL assays and digested with DNase I or exonuclease III (Boehringer Mannheim FRG) as described (Payvar et al., 1983; von der Ahe et al., 1985). All cleavage products and the corresponding Maxam–Gilbert sequencing reactions (Maxam & Gilbert, 1977) were analyzed on denaturing 8% (w/v) polyacrylamide gels and visualized by autoradiography.

Results

GR Purification. Crude cytosol prepared from a suspension volume of 10 L, corresponding to approximately 10^{11} HeLa S_3 cells, contains 70 ± 10 μg (mean ± SD; n = 6) of hGR as assessed by a dextran-coated charcoal assay. hGR was purified by sequential chromatography on phosphocellulose and DNA–cellulose (see Experimental Procedures). hGR was eluted from DNA–cellulose with 25 mM $MgCl_2$ (Figure 1) and applied on a DEAE-Sepharose column. The GR eluted at 0.11–0.12 M NaCl (Figure 2). The total recovery of purified hGR following the DEAE-Sepharose step was approximately 30 ± 5 μg (n = 6) (Table I). The purity of DEAE-Sepharose-purified 94K hGR was 50–60% according to densitometry scanning of SDS electrophoresis gels (Figure 3). In addition to the 94K GR band, there was always a 79K band present that constituted 10–20% of the GR preparation as measured by densitometry. Sometimes a 72K band as well as a 39K band was seen.

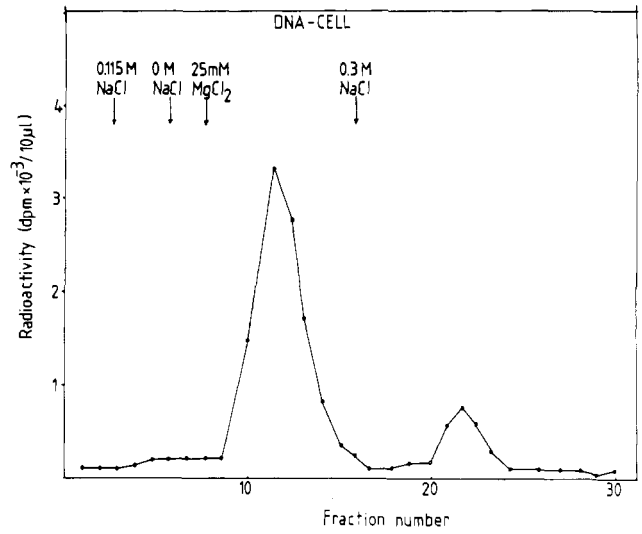

FIGURE 1: Chromatography of hGR on DNA–cellulose. The DNA–cellulose column was washed with 30 mL of buffer B containing 90 mM NaCl and subsequently with 20 mL of buffer B containing 115 mM NaCl. After a final wash with 20 mL of buffer B without NaCl, receptor was eluted with 30 mL of 25 mM $MgCl_2$ and 0.3 M NaCl, in the indicated order and in buffer B. For further details, see Experimental Procedures.

Table I: Purification of the [^{3}H]Triamcinolone Acetonide–Receptor Complex from 2 × 10^{11} HeLa S_3 Cells

	protein (mg)[a]	receptor fmol/mg	receptor μg[b]	purity (%)	purification (x-fold)
cytosol	900	1004	85	0.009	1.0
phosphocellulose	350	1823	60	0.017	1.8
DNA–cellulose	0.125	3824000	45	36	3808
DEAE-Sepharose, fractions 8–14 (cf. Figure 2)	0.040	6650000	25	62.5	6624

[a] Determined according to Lowry et al. (1951). [b] Determined by assuming one steroid molecule per receptor and a receptor molecular weight of 94 000.

The GR eluted from the DNA–cellulose column was analyzed by gel filtration on Sephadex G-200 (Figure 6) and by glycerol gradient centrifugation (Figure 7a). Gel filtration on calibrated columns gave a single peak with a Stokes radius of 5.8 nm (Figure 6). The sedimentation coefficient in 0.15 M NaCl was 4–4.4 S (Figure 7a). On the basis of these values, the approximate molecular weight of hGR weight was calculated to be 98 000–108 000 (assuming a partial specific volume of 0.732 cm^3/g and a solvation factor of 0.2 g of solvent/g of solute) (Sherman et al., 1983).

Scatchard Analysis and Ligand Specificity of the [^{3}H]TA Binding Site. Scatchard analysis (Scatchard, 1949) of binding data indicated the presence of a single class of high-affinity binding sites in HeLa S_3 cells. The apparent dissociation

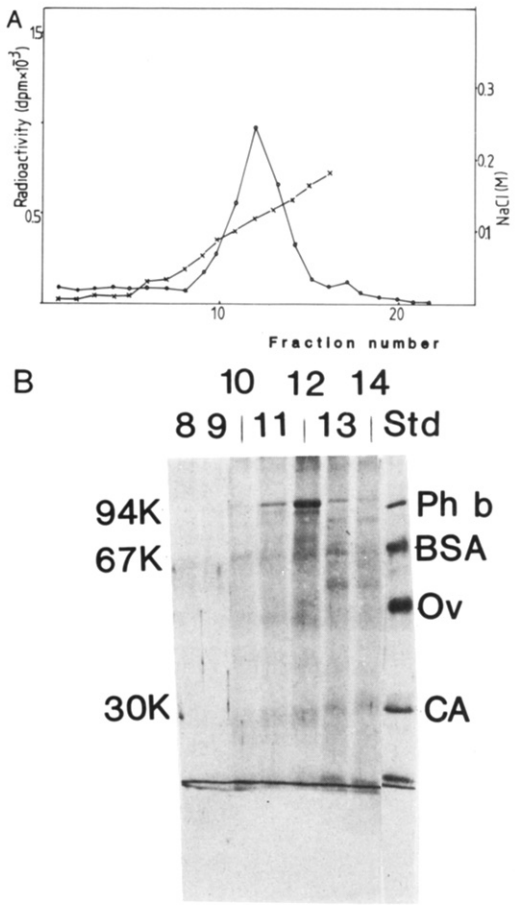

FIGURE 2: (A) DEAE-Sepharose chromatography of hGR eluted from the DNA–cellulose column by 25 mM $MgCl_2$. 50 μL/fraction was assayed for radioactivity (O). 0.5 mL of indicated fractions was analyzed by SDS gel electrophoresis, and 100 μL of indicated fractions was used for conductivity measurements (×). (B) Silver-stained 12% (w/v) SDS–polyacrylamide gel showing fractions 8–14 from the DEAE-Sepharose chromatography; Std, standard proteins (see Experimental Procedures). According to radioactivity analysis, the applied amount from fraction 12 corresponded to 0.80 μg of human GR. For further details, see Experimental Procedures.

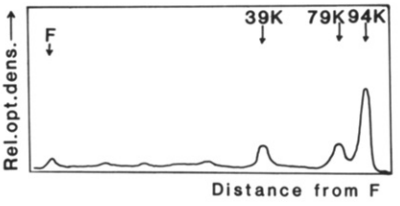

FIGURE 3: Densitometric scan of DEAE-Sepharose-purified hGR. hGR, 1 μg, was analyzed by SDS gel electrophoresis in a 12% (w/v) polyacrylamide gel and then silver stained and scanned in a Beckman R-112 densitometer at 550 nm. F signifies the electrophoretic front.

constant (K_D) for the hormone receptor interaction at 4 °C ranged between 4.5 and 6.5 nM. The number of cytosolic receptor sites per cell was about 30000 and the specific binding capacity of HeLa S_3 cytosol approximately 750 fmol of hGR/mg of protein (calculated from Scatchard plots; data not shown).

The competition of various steroids for glucocorticoid binding sites in HeLa S_3 cells using [^{3}H]TA as ligand in the presence of increasing concentrations of unlabeled steroid showed an order of binding affinities of triamcinolone acetonide > dexamethasone > corticosterone > progesterone, testosterone, and estradiol. Dexamethasone and to a certain extent corticosterone could displace specifically bound TA while other hormones such as progesterone, testosterone, and

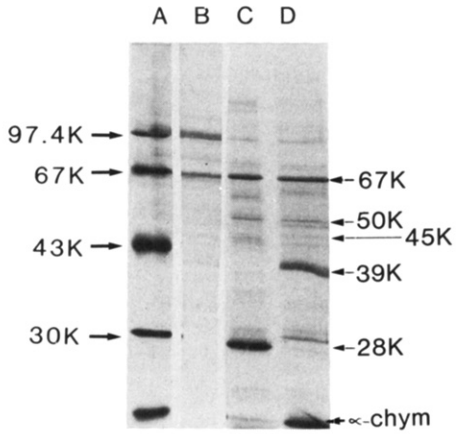

FIGURE 4: Trypsin and α-chymotrypsin (α-chym) digestion of DEAE-Sepharose-purified hGR as analyzed by SDS gel electrophoresis. Approximately 2 μg of hGR in a 400-μL volume was incubated with 0.8 μg of trypsin and α-chymotrypsin, respectively. Each sample was kept at 10 °C for 30 min and then cooled on ice and precipitated with trichloroacetic acid. Lane A, standard proteins (see Experimental Procedures); lane B, hGR; lane C, hGR + 0.8 μg of trypsin; lane D, hGR + 0.8 μg of α-chymotrypsin.

estradiol did not affect the specific binding of [^{3}H]TA (data not shown). The specificity of the binding sites for [^{3}H]TA strongly suggests that TA is bound to the GR in HeLa S_3 cell cytosol and that hGR exhibits similar hormone binding characteristics as the rGR.

Characterization of the 94K GR Component. Trypsin or α-chymotrypsin was used to study the proteolytic fragmentation pattern of DEAE-Sepharose-purified hGR as analyzed by SDS gel electrophoresis. Previously, the characteristic proteolytic digestion pattern of rGR has been studied (Gustafsson et al., 1984; Reichman et al., 1984). On the basis of these studies, it has been suggested that GR can be divided into three domains: a DNA binding, a steroid binding, and an antibody binding domain (Carlstedt-Duke et al., 1982). Treatment of hGR with α-chymotrypsin induced the formation of a major band corresponding to M_r 39000 and three minor bands corresponding to M_r 50000, 45000, and 30000, whereas the major band induced by trypsin digestion corresponded to M_r 28000. Trypsin digestion also induced the formation of two minor bands corresponding to M_r 50000 and M_r 45000 (Figure 4). *Staphylococcus aureus* V8 protease induced the formation of five major bands corresponding to M_r 61000, 55000, 50000, 40000, and 29000 for both the rat and human GR (data not shown). Taken together, the molecular weights of the major proteolytic hGR fragments obtained in this study are in good agreement with those previously reported for the purified hepatic rGR (Wrange et al., 1984).

Frozen GR preparations used for enzymatic digestion contained the GR degradation product corresponding to M_r 67000 (Figure 4). The 79K fragment was not seen when GR was digested with proteases at the concentration used in these experiments. Limited proteolysis with low concentrations of proteases or incubating GR for longer periods without proteases induced the formation of the 79K component (data not shown).

To determine which of the proteins are hormone binding, the receptor was covalently labeled with [^{3}H]TA by irradiation at 254 nm. Fractionation of this photoaffinity-labeled material by SDS–PAGE revealed a major peak of radioactivity corresponding to M_r 94K and a minor peak at 79K (Figure 5). The 79K component contained radioactivity equal to 15–20% of the amount of radioactivity associated with the 94K component. The 39K component was not possible to photoaffinity

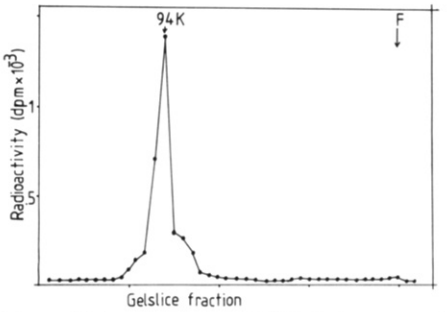

FIGURE 5: Photoaffinity labeling of DEAE-Sepharose-purified hGR. F signifies the electrophoretic front, and the number above the arrow signifies the molecular weight calculated from a parallel run on standard proteins (for further details, see Experimental Procedures).

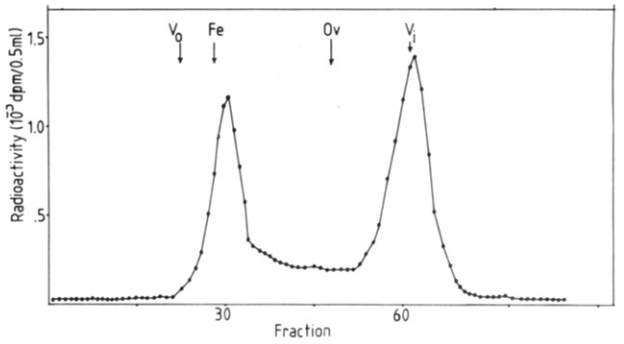

FIGURE 6: Sephadex G-200 chromatography of the hGR eluted from the DEAE-Sepharose column. 5 mL of purified receptor (~4 μg) was chromatographed on Sephadex G-200. The column was precalibrated with ferritin (Fe) and ^{14}C-labeled ovalbumin (Ov). The void volume was determined with blue dextran (V_0).

label by using this method, probably reflecting a too low concentration of this component in this particular preparation.

Reaction of Human GR with Anti-Rat Liver GR Antibody. Nine monoclonal antibodies raised against the hepatic rGR (Okret et al., 1984) were assayed by a second-antibody precipitation assay in order to select for antibodies cross-reacting with hGR.

Human GR from HeLa S_3 cells as well as from lymphocytes was labeled with either [^{3}H]TA or [^{3}H]dexamethasone. The reason for testing different ligands was our earlier observations indicating differences in cross-reactivity depending on the ligand used. In the second-antibody immunoprecipitation assay, we could not show reproducible cross-reactivity using TA as ligand (Okret et al., 1984) whereas the cross-reaction of the anti-rGR monoclonal antibodies to hGR was a reproducible phenomenon when dexamethasone was used. Thus, five antibodies out of nine cross-reacted with dexamethasone-labeled hGR from HeLa S_3 cells, human peripheral lymphocytes, and human skin and lung fibroblasts.

One monoclonal antibody, 7 (Okret et al., 1984), was tested for cross-reactivity with hGR by glycerol density gradient centrifugation and immunoblotting. The 4S cytosolic [^{3}H]-dex–GR complex from HeLa S_3 cells and from peripheral lymphocytes shifted to a sedimentation coefficient of 8.5 S in gradients containing 0.15 M NaCl when incubated with the antibody (Figure 7b), indicating the formation of an antibody–[^{3}H]dex–hGR complex.

The immunoblotting technique (Towbin et al., 1979) was used to test the monospecificity of the monoclonal antibody. A 50–60% pure hGR preparation (Figure 8A, lane 1) and a parallel sample to which 100 times the amount of cytosolic protein was added (Figure 8A, lane 2) were separated by SDS–PAGE. Parallel preparations were analyzed by immunoblotting using the monoclonal antibody 7 (Figure 8B, lanes

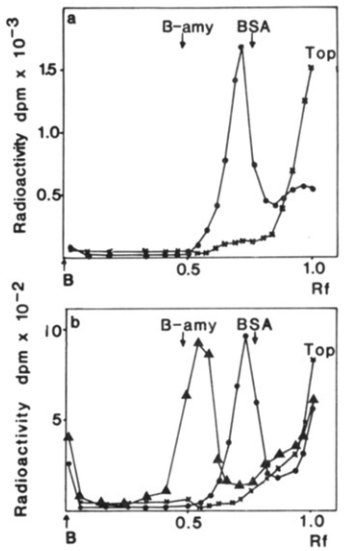

FIGURE 7: Reactivity of anti-rGR monoclonal antibodies with hGR. (a) Crude HeLa S_3 cytosol was incubated with [^{3}H]TA only (●) or with [^{3}H]TA in the presence of a 200-fold excess of unlabeled TA (×). (b) [^{3}H]TA–GR complex (0.4 pmol) from crude HeLa S_3 cytosol was incubated with 250 μL of ascites containing monoclonal antibody 7 (▲) and with 250 μL of control ascites, Sp 2/0 (●), for 2 h at 4 °C. (×) shows a parallel incubation of cytosol with [^{3}H]TA in the presence of a 200-fold excess of unlabeled TA. The incubation mixtures were layered on 12–50% (w/v) glycerol density gradients and centrifuged as described under Experimental Procedures.

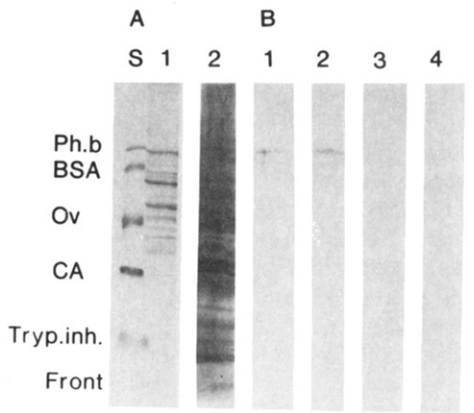

FIGURE 8: Immunoblotting of human HeLa S_3 cell cytosolic GR separated by SDS–PAGE. (A) Protein pattern after silver staining of the gel. (B) Electrophoretic transfer of the proteins to nitrocellulose sheets. S, standard proteins. HeLa S_3 GR from a 50% pure GR preparation (panel A, lane 1, and panel B, lanes 1 and 3) or from an identical hGR preparation to which 10 μg of cytosolic protein was added (final purity of hGR, 1.0%; panel A, lane 2, and panel B, lanes 2 and 4) was trichloroacetic acid precipitated and analyzed by 12% (w/v, polyacrylamide) SDS–PAGE as described under Experimental Procedures. Panel B shows incubation with ascites containing monoclonal antibody 7 (lanes 1 and 2) and incubation with ascites from Sp 2/0 cells which served as control (lanes 3 and 4).

1 and 2). Regardless of the purity of the GR preparation, the antibody detected two bands with mobilities (M_r 94 000 and 79 000) identical with that of the [^{3}H]TA photoaffinity-labeled hGR, indicating a high specificity for GR. The 94K band was detectable at a dilution of ascites of 1:2000. At a dilution of ascites of 1:250 and 1:500, and 79K band was observed. The 79K component is probably a receptor degradation product that copurifies with the intact 94K component (Westphal et al., 1982). No immunoreactivity was observed when identical GR preparations were incubated with ascites from Sp 2/0 cells (Figure 8B, lanes 3 and 4).

Binding of Purified hGR to GR Binding Sites in MTV. The

DNA binding properties of the purified hGR were investigated. To test if the hGR recognizes the same nucleotide sequences as rGR does, DNase I protection experiments were performed with MMTV. Defined DNase I footprints have been established for rGR by using the left-hand LTR region of MMTV (Payvar et al., 1983; Scheidereit et al., 1983). Previous experiments have indicated a higher affinity of the rGR for MMTV binding site 1:3 as compared to binding sites 1:4 and 1:5 (Payvar et al., 1983; von der Ahe et al., 1985). As shown in Figure 9B, similar concentrations of both purified rGR and hGR (7.5–15 nM) exhibited analogous binding activities. The highest degree of DNase I protection was observed for binding site 1:3 within binding region 1. Furthermore, very weak binding, if any, to binding site 1:4 was seen for both receptor preparations. Finally, bands in the region just downstream of binding site 1:3 are intensified in the presence of both hGR and rGR (Figure 9B) as has previously been shown (Payvar et al., 1983). These observations were further confirmed by exonuclease III protection experiments which showed several stops of the enzyme in the absence of receptor (Figure 9C), probably representing sequence-dependent interruptions in the exonuclease digestion (von der Ahe et al., 1985). The inhibition of exonuclease action by hGR was similar to what has been described for rGR (von der Ahe et al., 1985) i.e., a predominating stop at −190 which deliniates the border of binding site 1:3 and coincides with the limits of the DNase I footprints.

Discussion

In this study, we used a differential chromatography procedure on polyanionic matrices developed for the purification of the rGR (Wrange et al., 1979) in order to purify the human GR from HeLa S_3 cells. This procedure is based on the ability of the steroid–receptor complex to interact with DNA following an activation step. By slightly modifying the original purification procedure for rGR (Wrange et al., 1984), we have purified the hGR. By this purification procedure, we obtain a major component which is indistinguishable from the hepatic rGR with an approximate molecular weight of 94 000 as judged from SDS–PAGE. A 79K steroid binding component is also obtained and probably constitutes a receptor degradation product that copurifies with the intact 94K component (Wrange et al., 1984; Westphal et al., 1982). Consistent with this view, cross-reacting monoclonal antibodies raised against purified 94K protein from rat liver also reacted with the 79K component of hGR, as well as with the rGR 79K component. In addition, limited digestion with α-chymotrypsin cleaves both the 94K rGR and the 94K hGR to a 79K fragment. Alternatively, the 79K GR component may not represent a proteolytic degradation fragment but result from transcription initiated at an alternative start site localized 3′ to the conventional one (Miesfield, et al., 1986). The 72K component sometimes present in the GR preparations is an inconsistent finding and its biological significance unclear. It is resistant to proteolytic digestion by the proteases used in this study.

Numerous studies have reported purification and subsequent physicochemical characterization of the hGR by photoaffinity labeling of the receptor and by ion-exchange chromatography (Harmon et al., 1984; Bell et al., 1984). Although the hGR has been characterized in crude extracts from human lymphocytes (Murakami et al., 1979), human skin fibroblasts (Gadson et al., 1984; Hoschützky & Pongs, 1985), and HeLa S_3 cells (Currie & Cidlowski, 1982), no data exist on properties of purified hGR. In our study, hGR has been purified from HeLa S_3 cells to 50–60% homogeneity. These preparations have been used to investigate the physicochemical properties of the receptor and to study its specific DNA binding characteristics.

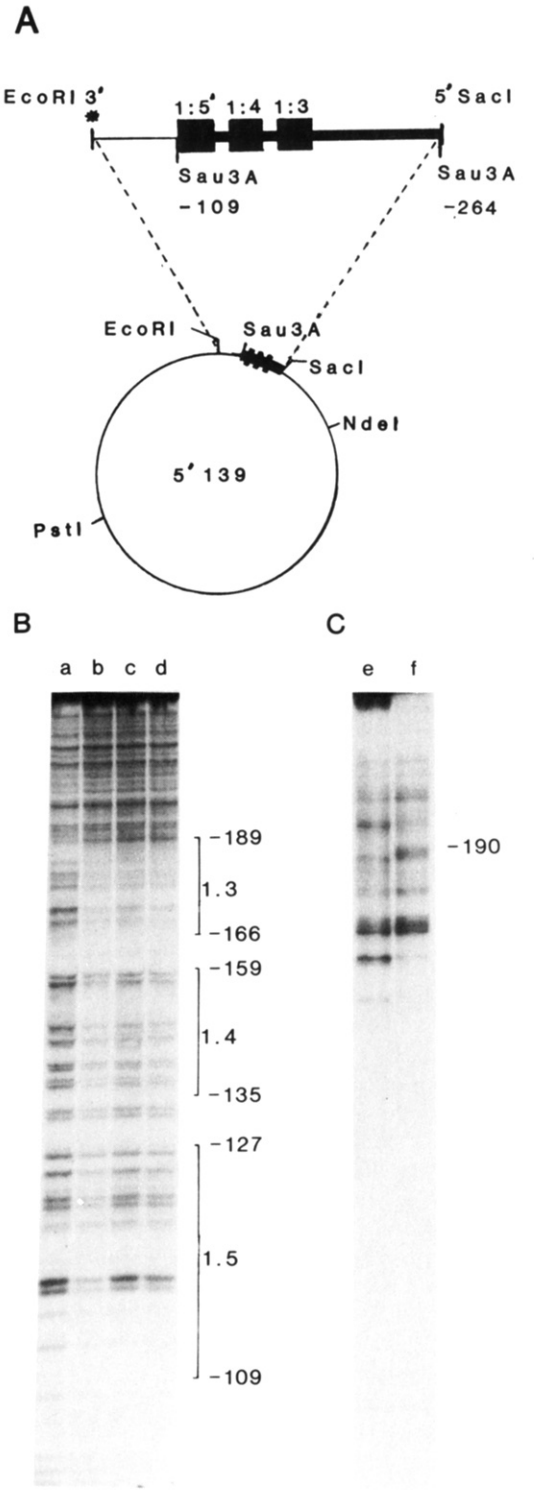

FIGURE 9: Binding of hGR to sequences in MMTV. A 185 bp *Sac*I–*Eco*RI fragment from pLS5′139 (A) containing MMTV sequences from positions −264 to −109 relative to the transcription start site was either 3′ labeled or 5′ labeled at the *Eco*RI site. The thick line represents the left-hand long terminal repeat of MMTV. Binding site 1:5′ represents the upstream half of the original binding site 1:5 (Payvar et al., 1983). Two nanograms of labeled fragment was incubated with varying concentrations of either purified rGR or hGR and subjected to digestion with (B) DNase I or (C) exonuclease III prior to analysis on 8% (w/v) polyacrylamide–urea gels. Lanes a and e, no protein; lane b, 150 ng of rGR; lane c, 100 ng of hGR; lanes d and f, 150 ng of hGR.

In this report, we demonstrate interspecies similarities between hGR and rGR. The evidence for these interspecies

similarities of GR is 4-fold: (1) monoclonal antibodies raised against the 94K rat hepatic GR cross-react with hGR from various tissues; (2) mild proteolytic digestion of purified GR from both species shows similar degradation patterns; (3) speicific DNA interactions, as assayed by DNase I and exonuclease III footprinting, are similar for rGR and hGR, indicating that these proteins might modulate gene activity in a similar way, by binding to the same specific DNA sequences; (4) the same purification procedure is applicable to both rGR and hGR, indicating that the receptor proteins have similar physicochemical properties.

Our results as well as results presented by Gametchu and Harrison (1984) and by Neatherhill and Bell (1984) confirm these observations and indicate that GR has some very well-conserved properties. Furthermore, a comparison between the rGR and hGR cDNA sequences (Hollenberg et al., 1985; Miesfeld et al., 1986) has revealed very few changes across the entire length of the polypeptide as well as highly homologous 3′-noncoding sequences which might be relevant for GR regulation (Okret et al., 1986).

Previously, it has also been concluded (Miesfeld et al., 1984, 1985) that the "modulatory domain" resides within the N-terminal half of the receptor protein and that the hormone binding domain resides near the C-terminus. The N-terminal domain corresponds to the domain previously shown to be immunoreactive (Carlstedt-Duke et al., 1982). Furthermore, this domain has been shown to contain the greatest variation in amino acid sequence (Miesfeld et al., 1986), thus giving rise to varying cross-reactivities with hGR using our anti-rGR monoclonal antibodies.

Hoschützky and Pongs (1985) postulated the existence of structural differences in the immunogenic domains of hGR and rGR. This was based on (a) differences in high-resolution ion-exchange chromatography properties of rGR and hGR, (b) lack of cross-reactivity for HeLa S_3 cell GR using one monoclonal mouse anti-rGR antibody (Westphal et al., 1982), and (c) different protease digestion patterns of rGR and hGR. In this study, however, several antibodies, but not all, raised against the hepatic rGR have been found to cross-react with the hGR as assayed by a second-antibody precipitation assay.

The reason for the differences in protease digestion patterns of rGR as compared to hGR found by Hoschützky and Pongs (1985) is unclear. However, the digestion experiments performed by Hoschützky and Pongs were carried out with crude extracts which may contain proteins able to interact with the receptor protein and/or the added proteases and thereby modify the proteolytic process. Furthermore, it is known that the receptor protein is more sensitive to proteolytic enzymes in very pure preparations as compared to crude extracts (Carlstedt-Duke et al., 1982).

At present, little is known about the molecular mechanism by which GR is able to modify the expression of specific genes. In this paper, we have observed similar protected nucleotide regions in MMTV DNA with the purified hGR and rGR, as studied by DNase I and exonuclease III protection assays, indicating close similarities in DNA binding properties of both proteins. It should, however, be mentioned that direct quantitative comparisons between hGR and rGR footprints are not informative, since the experiments involved different receptor preparations, and it is known that the specific DNA binding activity of GR may vary with different GR preparations (Payvar et al., 1983). In contrast to our observation, it has been suggested that the DNA binding ability of the progesterone receptor from different species to MMTV DNA may differ or be influenced by different proteins (Renoir et al., 1984; von der Ahe et al., 1986). In part, these differences might be due to different DNA binding activities of the so-called A (M_r 79000) and B (M_r 110000) components of the progesterone receptor found in various ratios in different species (Logeat et al., 1985). Clearly, studies with purified receptor preparations from different species will be of value in clarifying the binding of these proteins to specific DNA sequences.

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Identification and Amino Acid Sequence of the Deoxynucleoside Triphosphate Binding Site in *Escherichia coli* DNA Polymerase I†

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ABSTRACT: We have labeled the large fragment of *Escherichia coli* DNA polymerase I (Pol I) with pyridoxal 5′-phosphate, a substrate binding site directed reagent for DNA polymerases [Modak, M. J. (1976) *Biochemistry 15*, 3620–3626]. A covalent attachment of pyridoxal phosphate to Pol I results in the loss of substrate binding as well as the polymerase activity. The inactivation was found to be strictly dependent on the presence of a divalent metal ion. Four moles of pyridoxal phosphate was found to react per mole of the enzyme, while in the presence of substrate deoxynucleoside triphosphate only 3 mol of pyridoxal phosphate was bound. To identify the substrate-protected site on the enzyme, tryptic peptides from enzyme labeled with pyridoxal phosphate and tritiated borohydride, in the presence and absence of substrate, were resolved on a C-18 reverse-phase column. A single peptide containing the substrate-protected site was identified and further purified. The amino acid composition and sequence analysis of this peptide revealed it to span residues 756–775 in the primary acid sequence of Pol I. Lys-758 of this sequence was found to be the site of the pyridoxal phosphate reaction. It is therefore concluded that Lys-758 is the site of binding for the metal chelate form of nucleotide substrates in *E. coli* DNA polymerase I.

The general mechanism of the enzymatic synthesis of DNA has been elucidated, but the features of the molecular details of template-directed base selection and the basis for fidelity have not been clarified. Recent developments in the cloning of the enzymatically active large fragment (Klenow fragment) of *Escherichia coli* DNA polymerase I (Pol I)[1] (Joyce & Grindley, 1983), together with the knowledge of the primary amino acid sequence of entire Pol I (Joyce et al., 1982), have made the study of the structure–function relationship of this enzyme attainable. With the demonstration of high-resoluton

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; PLP, pyridoxal 5′-phosphate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; dNTP, deoxynucleoside triphosphate; $NaBH_4$, sodium borohydride; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

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