Association of the Glucocorticoid Receptor Binding Subunit with the 90K Nonsteroid-Binding Component Is Stabilized by both Steroidal and Nonsteroidal Antiglucocorticoids in Intact Cells†

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ABSTRACT: The interaction of various antiglucocorticoids with the glucocorticoid receptor from intact rat thymocytes was investigated. Reversible antiglucocorticoids (RU 486, cortexolone, progesterone) underwent more limited nuclear transfer than potent glucocorticoids (dexamethasone, triamcinolone acetonide, progesterone). This behavior was correlated with an impeded dissociation of cytosolic antiglucocorticoid receptor complexes preformed in intact cells, as assayed by high-performance size exclusion chromatography in physiological conditions (i.e., isotonic molybdate-free buffer). Antagonist–receptor complexes remained in a 7–8-nm form whatever the antiglucocorticoid tested (including dexamethasone mesylate and trifluoroperazine, a nonsteroidal antiglucocorticoid) and the incubation time at 37 °C, whereas agonist–receptor complexes were rapidly converted into 5-nm species. This stabilization was not detectable by conventional sucrose gradient centrifugation because of artifactual dissociation of untransformed complexes, a pitfall overcome by resorting to vertical tube centrifugation. Moreover, the low amount of nuclear antiglucocorticoid receptor complexes was also in the undissociated form, in contrast with nuclear agonist–receptor complexes. Immunological probes demonstrated that the 90-kDa non-steroid-binding component was associated with the antiglucocorticoid-stabilized receptor. Thus, whatever their chemical structure and their affinity for the receptor, antiglucocorticoids stabilize the oligomeric form of the glucocorticoid receptor in intact cells. Our data, demonstrating for the first time that all antiglucocorticoids probably act via a common mechanism, suggest a key role for subunit dissociation during in vivo receptor activation.

Since the description of the antiglucocorticoid activity of progesterone in rat thymocytes (Makman et al., 1967), many other steroidal (Chrousos et al., 1983) and nonsteroidal compounds (Van Bohemen & Rousseau, 1983; Loose et al., 1983) have been described as having antiglucocorticoid activity in various cellular models. However, no conclusive information could be drawn about the molecular mechanism of the glucocorticoid antagonist action at the receptor level from the first extensive reports devoted to the natural low-affinity antiglucocorticoids progesterone[1] and cortexolone (Rousseau et al., 1973; Turnell et al., 1974; Wira & Munck, 1974).

Significant advances in the understanding of antiglucocorticoid action could be expected following the recent description of RU 486, an optimal and highly potent antiglucocorticoid (Philibert, 1984). However, here again, published results appeared puzzling.

On one hand, some authors described RU 486 as being able to undergo limited but significant nuclear transfer in intact cells (Jung-Testas & Baulieu, 1983; Chasserot-Golaz & Beck, 1984; Coutard & Duval, 1985) and to display properties very similar to those of agonist–glucocorticoid receptor complexes in acellular extracts, including activation to the DNA-binding state (Schmidt, 1986; Agarwal et al., 1985), specific binding to glucocorticoid-regulated genes (Bourgeois et al., 1984; Willman & Beato, 1986), and in vitro transcription effects (Baulieu, 1987). On the other hand, we and others have found that RU 486 inhibited the activation of cytosolic glucocorticoid receptors (Moguilewsky & Philibert 1984; Sablonniere et al., 1986; Danze et al., 1987; Groyer et al., 1987) and was unable to yield in vivo footprints of glucocorticoid-regulated genes (Becker et al., 1986). Conflicting data were also reported about dexamethasone mesylate, a covalent marker of the glucocorticoid receptor displaying antiglucocorticoid activity (Miller et al. 1984; Richard-Foy et al., 1987).

If no clear picture emerged from these whole data, the few results obtained in intact cells were generally in better agreement with physiology than the ones obtained in acellular conditions. Therefore, we decided to develop a procedure allowing a rapid characterization of the native cellular receptor complexes and to apply it to various antiglucocorticoid derivatives.

This procedure involved the analysis of steroid–receptor complexes preformed in intact cells by high-performance size exclusion chromatography (HPSEC) in a physiological buffer containing no molybdate ions. Its use for the characterization

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[1] Abbreviations: Dex, dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; Dex Mes, dexamethasone 21-mesylate; TA, triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17α-acetonide; RU 486, 11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-propynylestra-4,9-dien-3-one; DXB, *N*-benzyl-9α-fluoro-16α-methyl-11β,17α-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxamide; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; cortexolone, 17α,21-dihydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; trifluoroperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HPSEC, high-performance size exclusion chromatography; SDS, sodium dodecyl sulfate; PAGE polyacrylamide gel electrophoresis; hsp, heat-shock protein; PMSF, phenylmethanesulfonyl fluoride; GR, glucocorticoid receptor.

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of RU 486 receptor complexes was described in a preliminary paper (Lefebvre et al., 1988). In the present study this experimental approach was applied to more than 10 natural and synthetic ligands of the glucocorticoid receptor, including six antiglucocorticoids of various chemical structure.

Our aim here was to determine if a common specific pattern of interaction of antiglucocorticoids with the receptor could be detected and related to unactivated receptor complex dissociation. If such a pattern could be observed, its relevance to the physiological steroid receptor activation process would probably be extremely high, since antiglucocorticoids, contrary to other compounds interfering with activation in vitro (e.g., molybdate), are biologically active in vivo.

Experimental Procedures

Materials. [1,2,4(n)-^{3}H]Dexamethasone (44 Ci/mmol), [1,2(n)-^{3}H]corticosterone (57 Ci/mmol), [1,2,4(n)-^{3}H]triamcinolone acetonide (28 Ci/mmol), and [1,2(n)-^{3}H] progesterone (40 Ci/mmol) were from Amersham, U.K. [1,2-^{3}H-(n)]deoxycorticosterone (41.8 Ci/mmol), [1,2,^{3}H(n)]-17α-hydroxy-11-deoxycorticosterone (53.3 Ci/mmol), and [6,7-^{3}H(n)]dexamethasone 21-mesylate (42 Ci/mmol) were from New England Nuclear (Boston, MA). [1,2,6,7-^{3}H]cortisol (100 Ci/mmol) was from C.E.A. (Saclay, France). [1,2,4-(n)-^{3}H]-*N*-Benzyl-9α-fluoro-16α-methyl-11β,17α-dihydroxy-3-oxo-1,4-androstadiene-17β-carboxamide (^{3}H DXB) was synthesized as described previously (Formstecher et al., 1980) from [^{3}H]dexamethasone and purified by reversed-phase high-performance liquid chromatography. Unlabeled trifluoroperazine was kindly provided by Prof. G. G. Rousseau. [6,7-^{3}H]RU 486 (50.6 Ci/mmol) and unlabeled RU 486 were obtained from Roussel Uclaf (Romainville, France). Other unlabeled steroids were purchased from Serva (Heidelberg, FRG). Monoclonal anti-Achlya 88-kDa protein antibody (Riehl et al., 1985) and GR 49/1 antiglucocorticoid receptor antibody (Westphal et al., 1982) were kindly provided by D. O. Toft and H. M. Westphal, respectively. Peroxidase conjugated anti-mouse IgG was from Institut Pasteur (Paris, France). Culture media were from Serva, and Ultroser SF was from Industrie Biologique Française (Villeneuve-la-Garenne, France).

Preparation and Labeling. Thymocytes were obtained from adrenalectomized male Wistar rats (180-g body weight) as described (Lefebvre et al., 1988). Briefly, thymus glands were removed, rinsed three times with calcium-free Hank's solution at 37 °C, and finely minced with scissors. Thymocytes were filtered through a nylon gauze and washed twice with Hank's solution. After overnight incubation in MEM Eagle's medium containing 2% Ultroser SF and 1% glutamine, adjusted to pH 7.4 with 20 mM Hepes, the cells were harvested and resuspended in serum-free MEM (or in Hank's buffer in case of further incubation in the presence of dexamethasone 21-mesylate) at 0.3×10^9 cells mL^{-1}. Incubation with tritiated steroids was then performed under gentle agitation for various times at 37 °C (unless otherwise mentioned) and was ended by centrifugation at 4 °C of the cell suspension. Overall cellular binding was assayed after the cell pellet was washed twice with ice-cold Hank's solution or by the rapid dilution assay of Munck and Wira (1975). Nonspecific binding was assayed in the presence of a 400-fold molar excess of unlabeled steroid.

Cellular Extract Preparation and Nuclear Transfer Assay. Steroid-treated cells were washed twice with ice-cold calcium-free Hank's buffer, 10^8 cells were homogenized in 0.25 mL of buffer A (16 mM potassium phosphate, 130 mM KCl, 20 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, and 10% glycerol), supplemented or not with 0.1% Triton X-100, by use of a Teflon–glass homogenizer. The homogenate was centrifuged at 4000*g* for 10 min. The supernatant was removed for radioactivity counting or further fractionation of the receptor complexes, whereas the crude nuclear pellet was washed twice in buffer A and then counted. In some experiments nuclear pellets were prepared according to the procedure of Munck and Wira (1975): incubated cells suspension was rapidly submitted to 50-fold dilution with 3 mM $MgCl_2$ at 4 °C, a procedure that resulted in complete cell disruption by hypotonic shock. A 4000*g* centrifugation was then performed for 10 min at 4 °C, and the nuclear pellet was assayed for radioactivity.

Purified nuclei were obtained by the Chauveau method (Chauveau et al., 1956). Incubated cells were resuspended in buffer B (20 mM TES, pH 7.4, 20 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 3 mM $MgCl_2$, 0.1% Triton X-100, 0.25 M sucrose, and 10% glycerol) and carefully homogenized in a loosely fitted Teflon–glass homogenizer. The homogenate was put onto a 2.2 M sucrose layer in buffer A and centrifuged for 45 min at 35000 rpm and 4 °C (Beckman SW 60 Ti rotor). Pellets containing intact purified nuclei were assayed for lactate dehydrogenase (by ultraviolet absorption spectrophotometric assay of NADH,H$^+$ at 340 nm) and DNA content (Burton, 1956). Morphological control of nuclei was performed by using light microscopy. Purified nuclei were washed twice in buffer A and then counted for radioactivity or submitted to extraction using buffer A supplemented with 0.4 M KCl or 50 mM $MgCl_2$.

Sucrose Gradient Centrifugation. Linear (5–20%) sucrose gradients were prepared in buffer A supplemented with 10% glycerol. Samples (0.2 mL) were layered onto the preformed gradients, and the tubes were centrifuged in a SW 60 Ti rotor (Beckman) at 55000 rpm and 4 °C for 16 h. Each tube was punctured, and 6-drop fractions were collected and counted for radioactivity. Sedimentation coefficients were determined according to the method of Siegel and Monty (1966) using catalase (11.2 S) and bovine serum albumin (4.3 S) as standards run in separate centrifugation tubes and detected by their absorbance at 280 nm. In some experiments a VTi 80 vertical tube rotor (Beckman) was used. In this case centrifugation time was 120 min at 60000 rpm.

High-Performance Size Exclusion Chromatography. The various molecular forms of the glucocorticoid receptor complexes were analyzed by chromatography on LKB TSK G3000 or TSK G4000 columns (7.5 × 300 mm) equilibrated in ice-cold buffer A without glycerol. Flow rate was 0.5 mL min^{-1} and pressure in the range 2.0–3.0 MPa. The chromatographic system was composed of a 6000 A solvent delivery device and a U6K injector equipped with a 2-mL sample loop (Waters, Milford, MA). Fractions (0.25 mL) were collected in a programmable Model 201 collector (Gilson, France) and assayed for tritium content or further incubated with [^{3}H]-triamcinolone acetonide, according to whether the samples submitted to HPSEC were already labeled with a tritiated ligand or not. In some cases aliquots of fractions were also submitted to SDS–PAGE analysis. Stokes radius calibration of the HPSEC columns was performed by using the following standards: thyroglobulin (8.5 nm), β-galactosidase (6.9 nm), ferritin (6.1 nm), catalase (5.2 nm), and aldolase (4.8 nm). The calibration curve of R_S versus $K_{av}^{1/3}$ was plotted according to the method of Porath (1963).

Determination of Binding Sites. Glucocorticoid receptor content of unlabeled HPSEC fractions was determined as follows: 200-μL aliquots of each fraction were incubated at

Table I: Comparative Binding of [^{3}H]RU 486 and [^{3}H]TA to Intact Rat Thymocytes and Rat Thymus Cytosol

	intact thymocytes				thymus cytosol	
	K_D[a] (nM)	N[a] (sites/cell)	B_S/B_T,[b] excess RU 486	B_S/B_T,[c] excess TA	K_D[a] (nM)	N[a] (fmol/mg of protein)
[^{3}H]RU 486	4.25 ± 0.90[d] (n = 3)	5600 ± 600 (n = 3)	0.76	0.48	0.93[d]	620
[^{3}H]TA	8.50 ± 0.40[d] (n = 2)	2900 ± 300 (n = 2)	0.64	0.64	1.52[d]	632

[a] Specific binding assayed in conditions (b) for [^{3}H]RU 486 and (c) for [^{3}H]TA. [b] B_T is total bound tritiated steroid and B_S specific binding calculated from $B_S = B_T - B_{NS}$, where B_{NS} was assayed in the presence of a 500-fold excess of unlabeled RU 486. Results are for typical incubations at a 30 nM [^{3}H]steroid concentration. [c] Same as (b) when using unlabeled TA. [d] The striking differences in K_D between cell-free and intact cell assays could be explained by the difference of temperature (0–4 vs 37 °C) between the two assays.

4 °C with 30 nM [^{3}H]triamcinolone acetonide in the presence or absence of a 200-fold excess of the same unlabeled steroid. All samples were supplemented with 1 mg mL^{-1} bovine serum albumin. After a 5-h incubation, the bound radioactivity was determined in duplicate by a charcoal adsorption assay.

Covalent Labeling. Covalent binding of dexamethasone 21-mesylate to macromolecules was determined by trichloroacetic acid (TCA) precipitation: to 125-μL aliquots of each fraction was added the same volume of 25% TCA. Each fraction was filtered through a nitrocellulose filter that was then assayed for radioactivity.

Gel Electrophoresis and Immunoblotting Procedures. SDS–polyacrylamide gel electrophoresis was performed in 7.5% acrylamide gel according to the method of Laemmli (1970). Molecular weight standards (Bio-Rad) were M_r 200 000 (myosin), 116 250 (β-galactosidase), 97 000 (phosphorylase *b*), 66 000 (bovine serum albumin), and 43 000 (ovalbumin).

Samples (10–80 μL) containing 0.1–20-μg markers or fractionated cytosol were layered on the gel with bromophenol blue as tracking dye, and electrophoresis was carried out at room temperature for 18 h. After electrophoresis, the gel slab was immediately blotted by using a LKB 2005 Transphor electroblotting unit (Bromma, Sweden) onto a nitrocellulose sheet (Towbin et al., 1979) that was then stained with 0.2% Ponceau red in 3% trichloroacetic acid. Sample and control tracks were cut out, and the former were saturated with a 5% solution of fatty acid free milk powder and then washed, incubated with the appropriate antibodies, and finally revealed with peroxidase-conjugated anti-mouse IgG antibodies (Institut Pasteur, Paris).

Miscellaneous. Radioactivity was determined in an LKB 1214 spectrometer using Optiphase (LKB, Bromma, Sweden) as scintillation cocktail (50% tritium efficiency).

RESULTS

Nuclear Transfer of Glucocorticoids and Antiglucocorticoids. Estimation of the nuclear transfer of tritiated steroids is subjected to considerable variations according to the experimental conditions used. Addition of a nonionic detergent like Triton X-100 to the nuclei preparation buffer permits nuclei to be washed free of cytoplasmic contaminants and selective removal of external nuclear membranes (Dwyer & Blobel, 1976; Holtzman et al., 1966). We have therefore assayed the nuclear transfer of various ligands of the glucocorticoid receptor in the presence or absence of Triton X-100. Detergent treatment did not significantly affect the stability of cytosolic TA and RU 486 receptor complexes: only 5–13% loss of specific binding sites was observed after a 60-min exposure to 0.1% Triton X-100 (data not shown). Moreover, no significant change of the apparent nuclear transfer of triamcinolone acetonide, dexamethasone, progesterone, and cortexolone was observed at this detergent concentration

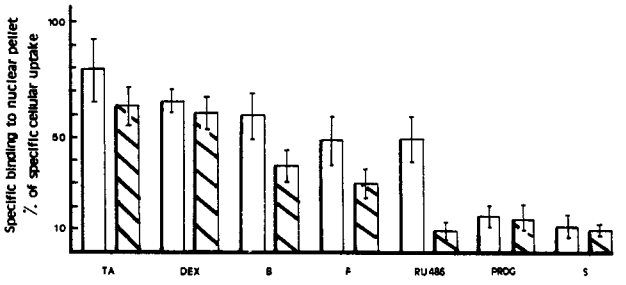

FIGURE 1: Nuclear transfer of various steroids in thymocytes. Cells were incubated for 30 min at 37 °C with several synthetic and natural glucocorticoid agonists and antagonists at a saturating concentration (20–600 nM according to the compound tested). After cell homogenization, specific binding to nuclear pellet was assayed either in the absence (open bars) or in the presence (marked bars) of 0.1% Triton X-100. Nonspecific binding was determined in parallel samples supplemented with a 100-fold molar excess of unlabeled steroid. Each value represents the average of three independent experiments and is expressed as the ratio of specific nuclear binding on specific overall cellular uptake of the steroid. (TA) Triamcinolone acetonide; (Dex) dexamethasone; (B) corticosterone; (F) cortisol; (Prog) progesterone; (S) cortexolone.

(Figure 1), which was reported to be unable to extract nuclear glucocorticoid receptor complexes (Kaufmann et al., 1982). On the contrary, Triton X-100 decreased the apparent nuclear transfer observed for corticosterone, cortisol, and RU 486. The moderate reduction found in the case of the former ligands remains unexplained (see also Munck and Holbrook (1984)], whereas the striking decrease of RU 486 nuclear transfer was due to the occurrence in intact cells of some additional RU 486 binding sites, distinct from the glucocorticoid receptor (Table I). While [^{3}H]TA binding sites appeared highly competitive by both unlabeled TA and RU 486, cellular [^{3}H]RU 486 binding sites were only partly occulted by an excess of unlabeled TA and outnumbered TA binding sites in a 1.6 to 2.0 ratio.

Since these additional RU 486 binding sites were not found in thymocytes cytosol, where binding sites were found in equal amounts for the two steroids and were mutually competitive, a nuclear or membranous localization could be attributed to these non-GR RU 486 binding sites. Subcellular fractionation on discontinuous sucrose layer and nuclei purification through a 2.2 M sucrose layer allowed us to demonstrate a nuclear localization for these sites (data not shown). Thus, Triton X-100 sensitive [^{3}H]RU 486 binding sites were probably located on the external nuclear membranes and clearly different from GR.

Therefore, the GR-specific nuclear transfer values obtained with 0.1% Triton X-100 were considered to be the most relevant ones. In these conditions, agonists underwent a high nuclear transfer ranging from 40 to 75%, according to the experiments. Similar variations in nuclear transfer assay have already been stressed by others (Munck & Holbrook, 1984). Cortisol, a not so highly efficient agonist in the rat (Kaiser

Table II: Hydrodynamic Parameters and Calculated M_r of the [^{3}H]RU 486, [^{3}H]Dex, and [^{3}H]TA Cytosolic Glucocorticoid Receptor Complexes[a]

steroid	Na_2MoO_4[b] (mM)	R_S (nm)	$s_{w,20}$	M_r
RU 486	20	7.2–8.3	8.2	270
	0	7.2–8.3[c]	4.6[e]	150
	0	7.2–8.3[d]	ND	
	0	7.2–8.3	8.2 and 4.6[f]	270 and 150
Dex or TA	20	5.0	4.0[e]	84
	0	5.0	4.0[e,f]	84

[a] Rat thymocytes were incubated for 30 min at 37 °C in the presence of 20 nM [^{3}H]steroid. Cytosol was then rapidly prepared in buffer A containing 1 mM PMSF and 10% glycerol and analyzed by both HPSEC on a TSK G3000 SW column and sucrose gradient centrifugation for R_S and $s_{w,20}$ determination, respectively. Sucrose gradient centrifugation was performed by using either a conventional swinging-bucket rotor (SW 60 Ti, Beckman) with a 17-h centrifugation time at 55 000 rpm or a vertical tube rotor (VTi 80, Beckman) with a 120-min centrifugation time at 60 000 rpm. Apparent molecular weight M_r was calculated according to the formula $M_r = 4224 R_S s_{w,20}$. ND = not determined. [b] In the buffer used for the HPSEC and sucrose gradient assays. [c] Analysis performed 20 min after cell homogenization. [d] Analysis performed 24 h after cytosol preparation and conservation at 0 °C. [e] Analysis performed by using conventional 17-h sucrose gradient centrifugation. [f] Analysis performed by using a 120-min centrifugation in a vertical tube rotor. About 80% of the bound radioactivity was found in the 8.2-S peak obtained with RU 486, whereas no occurrence of the 8.2-S peak was observed with triamcinolone acetonide.

et al., 1972), was able to only induce a 29% nuclear transfer. Contrasting with agonists, antagonists (RU 486, progesterone, cortexolone) underwent low nuclear transfer, with values ranging from 5 to 15%. Thus, resorting to the nonionic detergent Triton X-100 led to data showing a good qualitative correlation between nuclear transfer and agonist potency. The only exception was dexamethasone 21-mesylate, with which a 53% nuclear transfer was observed. However, in this case, more than 75% of the nuclear radioactivity remained unextractable in condition where 60–70% of radioactive agonists were solubilized and no glucocorticoid receptor complexes could be characterized in the nuclear extract, suggesting that Dex-Mes was nonspecifically bound to proteinaceous components of nuclei.

Hydrodynamic Parameters of [^{3}H]RU 486– and [^{3}H]-Dexamethasone–Receptor Complexes. Cytosolic extracts of thymocytes incubated for 30 min at 37 °C with either [^{3}H]RU 486 or [^{3}H]dexamethasone were analyzed in parallel by sucrose gradient centrifugation and HPSEC performed in an isotonic buffer supplemented or not with 20 mM sodium molybdate (Table II). Whatever the conditions used, HPSEC yielded a 7.2–8.3-nm Stokes radius (R_S) for RU 486–glucocorticoid receptor complexes and a 5-nm R_S for dexamethasone–glucocorticoid receptor complexes. Thus, the antiglucocorticoid RU 486 clearly stabilized a high molecular size receptor complex in isotonic conditions even in the absence of molybdate. This form appeared to be rather stable in the native cytosol at 0 °C. On the other hand, conflicting results were provided by sucrose gradient sedimentation experiments: 4.6-S GR–RU 486 complexes were observed in the absence of molybdate, whereas a 8.2-S value was obtained in presence of 20 mM Na_2MoO_4, in sharp contrast with the 4.0-S value always obtained with both the optimal agonists tested, i.e., dexamethasone and triamcinolone acetonide (Lefebvre et al., 1988). Since the in vitro 8S to 4S conversion of steroid glucocorticoid receptor complexes is an irreversible phenomenom in the conditions used in our experiments, regardless of the presence or absence of molybdate in the sucrose gradient buffer, we concluded that the low 4.6-S value obtained after the time-consuming sucrose gradient centrifugation (16 h) probably resulted from an artifactual size reduction by subunit dissociation during the centrifugation step. This hypothesis was confirmed by sucrose gradient analysis performed in a vertical tube rotor, which allowed a reduction of the centrifugation time: after 120 min most [^{3}H]RU 486–receptor complexes remained in the 8.2S form with only limited dissociation to the 4.6S one (0–50% according to the experiments). Such a dissociation did not take place during the very rapid (less than 20 min) HPSEC assay, which therefore appeared particularly well suited for the fast characterization of steroid receptor complexes preformed in intact cells.

Effect of the Ligand on the Kinetics of the Size Reduction of Glucocorticoid Receptor Complexes in Intact Cells. Our preliminary observation (Lefebvre et al., 1988) of a time- and temperature-dependent size reduction of agonist glucocorticoid receptor complexes preformed in intact cells and rapidly assayed in cytosolic extracts by high-performance size exclusion chromatography was confirmed. When thymocytes were incubated with full agonists like dexamethasone, triamcinolone acetonide, and corticosterone (the physiological glucocorticoid agonist in the rat), the 7–8-nm form of the receptor was the only one found after a short 5-min incubation of 37 °C, whereas prolongation of the exposure of intact cells to the steroid at the same temperature led to a rapid conversion of the receptor complexes toward a lower size form eluted in the 4.0–5.0-nm region of the chromatogram (Figure 2). This conversion, complete in less than 15 min, followed kinetics very similar to the ones observed for the nuclear transfer of the steroid (not shown) and was therefore supposed to be concomitant with receptor activation. Further support of this hypothesis was afforded by the observation that both receptor dissociation to the 5-nm form and nuclear transfer were almost completely inhibited when cells were incubated with agonists at 4 °C. Moreover, here again, cortisol displayed a particular pattern with only a partial 50–70% conversion of the receptor complexes to the 5-nm form after 30 min at 37 °C, a picture in good agreement with both its moderate nuclear transfer and rather low agonist agreement with both its moderate nuclear transfer and rather low agonist activity in the rat (Figures 1 and 2).

In contrast with glucocorticoid agonists, antiglucocorticoids only generated 7–8-nm receptor complexes and appeared unable to provoke any shift toward the 5-nm form whatever the incubation time at 37 °C (up to 6 h). This result was consistently observed with the six steroidal antiglucocorticoids tested (Figure 3): RU 486; DXB (a 17β-carboxamide derivative of dexamethasone (Rousseau et al., 1979); dexamethasone 21-mesylate; 11-deoxycorticosterone, a partial agonist displaying antiglucocorticoid activity at 40 nM concentration (Colbert & Young, 1986); cortexolone; and progesterone. Control incubation in the presence of an excess of unlabeled dexamethasone allowed the discrimination of any binding to macromolecular components distinct from the glucocorticoid receptor. Thus, steroidal antiglucocorticoids, irrespective of the particular chemical features responsible for their antagonist activity, which might stem from a modification on the 11-position (RU 486, deoxycorticosterone, cortexolone, and progesterone) or on the 17-position side chain (DXB and dexamethasone mesylate), shared a similar ability to stabilize the nonactivated glucocorticoid receptor.

Stabilization of the 7–8-nm Form of the Glucocorticoid Receptor by a Nonsteroidal Antiglucocorticoid. The results obtained with steroidal antiglucocorticoids prompted us to check if trifluoroperazine, a nonsteroidal antiglucocorticoid in rat hepatoma cells (Van Bohemen & Rousseau, 1982), was

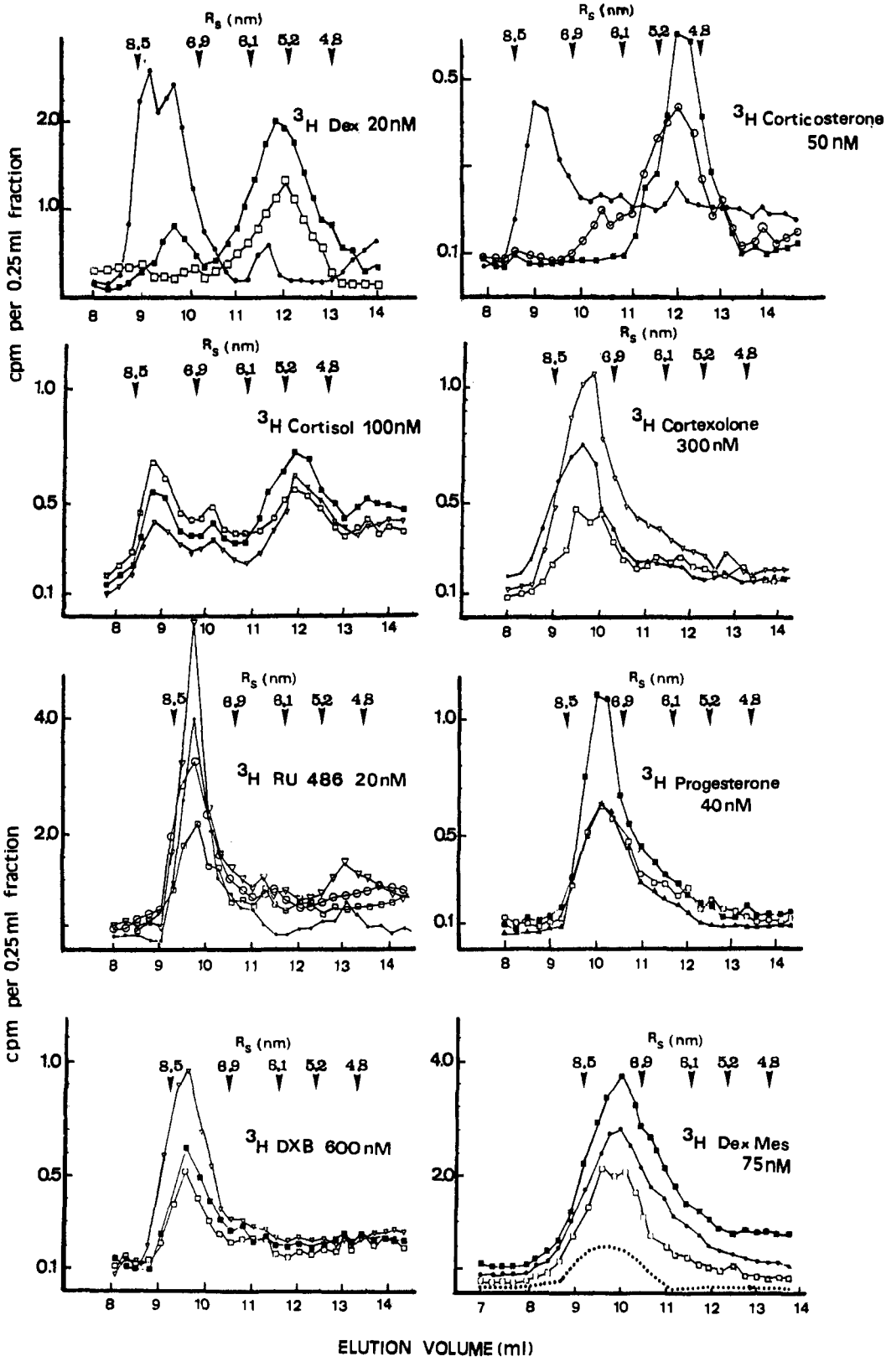

FIGURE 2: Size reduction kinetics of glucocorticoid receptor complexes in intact cells. Thymocytes were incubated in the presence of different tritiated steroids at the indicated concentrations, and after various times, cells were rapidly homogenized to prepare 4000*g* supernatants that were analyzed by HPSEC G4000 SW column. The entire procedure needed less than 30 min. Data were corrected for the nonspecific binding assayed in control incubations in the presence of a 100-fold excess of the respective unlabeled steroid except in the case of dexamethasone mesylate and cortexolone, where unlabeled dexamethasone was used. The elution pattern of bound [^{3}H]deoxycorticosterone (data not shown) was closely similar to the one obtained with progesterone. The decreasing height of the 7–8-nm peaks observed with antiglucocorticoids was mainly related to some variation in sample recovery after the iterative injections needed for kinetic studies but comprising a probably too short washing between consecutive injections. Incubation times were as follows: (●) 5 min; (▽) 15 min; (■) 30 min; (○) 45 min; (□) 60 min. For Dex Mes, the dotted line represents trichloroacetic acid precipitable binding activity. According to this assay, 27% of the cytosolic glucocorticoid receptor complexes were covalently labeled after a 60-min incubation. Data for Dex and RU 486 are the same as previously reported (Lefebvre et al., 1988).

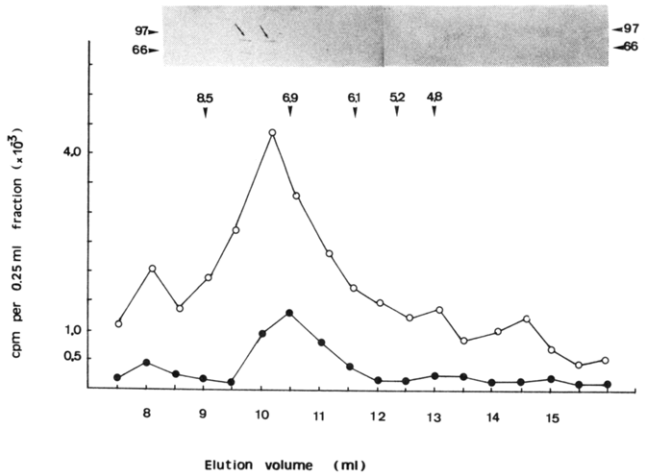

FIGURE 3: Analysis of trifluoroperazine–glucocorticoid receptor complexes by exchange assay and Western blot analysis. Intact thymocytes were incubated for 30 min at 37 °C in the presence of either 6 μM trifluoroperazine or 30 nM unlabeled RU 486. Cytosols were then prepared, and 0.25-mL samples were submitted to HPSEC analysis on a TSK G4000 SW column. Fractions (0.5 mL) were collected at 4 °C and 0.2-mL aliquots immediately incubated for 5 h in the presence of 30 nM [^{3}H]TA either alone or supplemented with a 400-fold molar excess of unlabeled TA. Bound radioactivity was assayed by using dextran-coated charcoal. Specific binding was plotted versus elution for both trifluoroperazine (○) and RU 486 (●) treated thymocytes. One-tenth milliliter of each HPSEC fraction of the trifluoroperazine-treated sample was analyzed by Western blot using GR 49 antireceptor antibody. Arrows indicate the position of molecular weight standards: phosphorylase *b* (97 000) and bovine serum albumin (66 000).

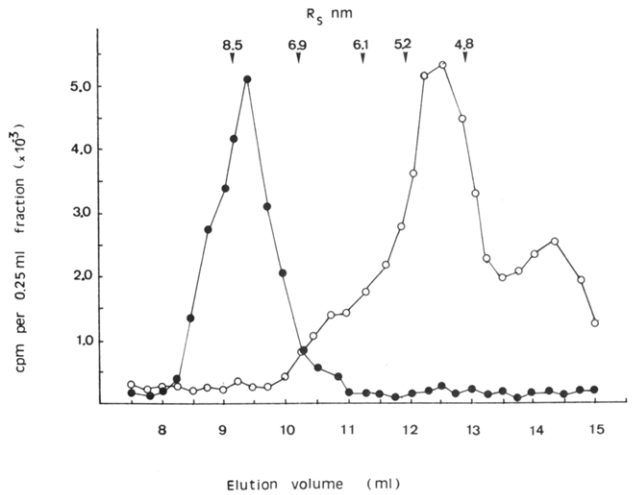

FIGURE 4: HPSEC analysis of nuclear glucocorticoid receptor complexes. Nuclei of thymocytes previously incubated for 120 min at 37 °C in the presence of 20 nM of either [^{3}H]RU 486 or [^{3}H]TA were purified through a 2.2 M sucrose layer. Purified nuclei were then washed twice with buffer A and extracted for 30 min at 4 °C with buffer supplemented with 50 mM $MgCl_2$. The soluble fractions of the extracts were injected on a TSK G4000 SW column. Radioactivity in the effluent was plotted against elution volume for both [^{3}H]RU 486 (●) and [^{3}H]TA (○) treated samples. RU 486–glucocorticoid receptor complexes were extracted from a nuclear fraction containing 3.78 mg of DNA and 2% residual lactate dehydrogenase activity (when compared to whole cellular homogenate), whereas TA–glucocorticoid receptor complexes were obtained from a fraction containing 1.72 mg of DNA and 4% residual lactate dehydrogenase activity. Column calibration was performed with usual size markers.

also able to inhibit the size reduction of the glucocorticoid receptor complexes provoked by glucocorticoid agonist in intact cells. However, since trifluoroperazine was only available in unlabeled form, the study of its effect on the glucocorticoid receptor complex size required an exchange assay of the receptor. Therefore, postelution labeling of the HPSEC fractions was performed with [^{3}H]TA. Starting from thymocytes previously incubated in the presence of either trifluoroperazine or unlabeled RU 486, significant glucocorticoid receptor activity was recovered after HPSEC, but only in the 7–8-nm region of the chromatogram (Figure 3). The much higher affinity of RU 486 (K_d = 0.9 nM) than of trifluoroperazine (K_d = 6 μM in rat hepatoma cells; Van Bohemen & Rousseau, 1982) for the glucocorticoid receptor probably accounted for the higher exchange rate and therefore higher binding activity recovery obtained with trifluoroperazine. On the contrary, after thymocytes were incubated in the absence of any ligand of the receptor, no [^{3}H]TA binding could be detected in any HPSEC fraction. Since there is evidence that activated glucocorticoid receptors are not detected by exchange assay (Noguchi et al., 1987, and unpublished data), we submitted the HPSEC fractions to Western blot analysis using an antireceptor antibody (Westphal et al., 1982). In trifluoroperazine-treated thymocytes receptor immunoreactivity was exclusively found in the 7–8-nm region, in perfect agreement with the exchange assay. In thymocytes incubated in the absence of any ligand, the receptor immunoreactivity was found in the 6-nm region (not shown).

Characterization of Nuclear Receptor Complexes. Thymocytes previously incubated for 120 min at 37 °C with [^{3}H]RU 486 or [^{3}H]TA were homogenized in the presence of 0.1% Triton X-100, and their nuclei were purified. The nuclear glucocorticoid receptor complexes were then extracted by 50 mM $MgCl_2$. Magnesium ions have been shown to remove glucocorticoid receptor complexes from both DNA–cellulose and cell nuclei (Carlstedt-Duke et al., 1984) and to have no receptor-activating property at this concentration, contrary to 0.4 M KCl (data not shown). Therefore, $MgCl_2$ allowed us to avoid the addition of molybdate in the extraction buffer in order to "freeze" activation of the nuclear receptor complexes. Extraction rates of receptor complexes by KCl or $MgCl_2$ were quite similar, with typical values around 60–70% of total nuclear bound radioactivity.

As expected, nuclear [^{3}H]TA complexes displayed a 5-nm R_S. On the contrary, nuclear [^{3}H]RU 486 receptor complexes were recovered as a homogeneous 8.3-nm peak (Figure 4). Quantitative exploitation of the HPSEC data revealed that the amount of nuclear receptor extracted from cells incubated with [^{3}H]RU 486 was only 26% of the amount obtained when [^{3}H]TA was used, a result in perfect agreement with nuclear transfer data. Finally, when $MgCl_2$ receptor extraction by HPSEC analysis was performed on 4000*g* nuclear pellets, the same results were obtained with RU 486, progesterone, and deoxycorticosterone: whatever the antiglucocorticoid used, all the solubilized nuclear receptor complexes were in the 8.3-nm form with no occurrence of any activated 5.0-nm complexes. Similar experiments were done with thymocytes incubated with 75 nM dexamethasone mesylate. Although $MgCl_2$ nuclear extracts contained 22% of the nuclear bound radioactivity, no specific binding could be characterized in any HPSEC fraction. Similar results were already reported for HTC cells (Simons et al., 1983).

Subunit Composition of Antiglucocorticoid Receptor Complexes. In order to establish the molecular composition of the 7–8-nm receptor complexes, we used two monoclonal antibodies: GR 49, which recognizes the native steroid binding subunit of both activated and unactivated molybdate-stabilized glucocorticoid receptor (Housley et al., 1985), and AC 88, a mouse anti-Achlya 88-kDa protein IgG1 (Riehl et al., 1985) usable as probe for the 90-kDa mammalian heat-shock protein

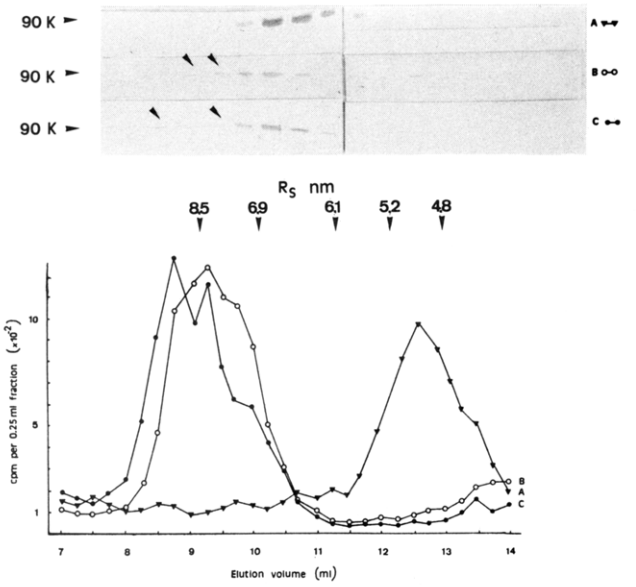

FIGURE 5: Coelution of antiglucocorticoid receptor complexes and AC 88 immunoreactive 90-kDa protein after HPSEC fractionation. Thymocytes were incubated at 37 °C with [^{3}H]TA or [^{3}H]RU 486, and cytosol extracts were then prepared and analyzed by HPSEC on a TSK G4000 SW column as described in Figure 3. Fractions were assayed for radioactivity content and submitted to Western blot analysis using the AC 88 antibody cross-reacting with mammalian steroid receptors associated 90-kDa protein (10, 52). Elution profiles A, B, and C correspond respectively to thymocytes incubated with [^{3}H]TA, [^{3}H]RU 486, and [^{3}H]RU 486 with treatment of the cytosol sample with antireceptor antibody GR 49 (53) at a 1/10 dilution overnight at 4 °C before HPSEC. Lanes A, B, and C show the 90-kDa region of the Western blot assay performed with AC 88 antibody on the respective cytosols after HPSEC fractionation. Size markers were thyroglobulin (8.5 nm), β-galactosidase (6.9 nm), ferritin (6.1 nm), aldolase (5.2 nm), and catalase (4.8 nm).

(hsp 90) associated to 8S unactivated steroid receptors (Sanchez et al., 1987; Schuh et al., 1985). Figure 5 depicts the hsp 90 repartition in HPSEC fractionated cytosols. In samples obtained from cells incubated in the presence of agonists, while receptor complexes displayed a 5-nm Stokes radius (curve A), the bulk of AC 88 immunoreactive material was mainly located in the 6–7-nm region, in good agreement with the reported dimeric structure of free hsp 90 (Schuh et al., 1985). In contrast, when the cells were incubated with an antagonist like RU 486 (elution profile B) or progesterone (data not shown), a small part of the AC 88 immunoreactive material was displaced toward the 8-nm region (see arrows in lane B). More strikingly, the shift of the [^{3}H]RU 486 receptor complexes from 7–8 to 8–9 nm provoked by the incubation of cytosol samples with the GR 49 antibody before the chromatographic step (curve C) was accompanied by a concomitant shift of the AC 88 immunoreactive material toward the same HPSEC fractions (lane C). Indeed, we checked that GR 49 had no effect on the HPSEC pattern of the free 90-kDa protein. Thus, the 7–8-nm glucocorticoid receptor complexes selectively stabilized by antiglucocorticoids in physiological conditions contain at least two distinct proteins, the steroid binding subunit and a 90-kDa AC 88 immunoreactive protein, which is very probably the hsp 90 described by others.

Discussion

Studies on the nuclear transfer of glucocorticoid receptors and the role of the steroid ligand in this phenomenon have generated controversial data. The technical conditions of the nuclear transfer assays performed with various antiglucocorticoids needed to be questioned in order to explain the striking quantitative differences reported among various cellular models using either progesterone, cortexolone, or RU 486 (Rousseau et al., 1973; Chasserot-Golaz & Beck, 1984; Svec & Harrison, 1979). For the latter compound results ranged from almost no nuclear translocation in rat thymocytes (Moguilewsky & Philibert, 1984) to near 50% translocation in mouse fibroblasts (Jung-Testas & Baulieu, 1983). Our data clearly show that the occurrence of some extra binding sites, distinct from the receptor, may give rise to misleading values and that a careful assay of cellular binding sites and the preparation of purified nuclei are required to obtain reliable results. By use of Triton X-100, a good qualitative relationship could be established between nuclear transfer and biological activity. Moreover, further characterization of the small amount of nuclear antiglucocorticoid receptor complexes found in nuclei demonstrated that antiglucocorticoids stabilized unactivated high-sized receptor complexes in intact cells whatever their apparent cytosolic or nuclear localization. Therefore, nuclear localization would not be equivalent to receptor activation to the DNA-binding state, as recently suggested by Picard and Yamamoto (1987). The actual localization of the unliganded glucocorticoid receptor and the hormone dependence of its nuclear transfer remain controversial issues (Welshons et al., 1985; Lukola et al., 1985).

Our demonstration that, whatever their chemical structure and affinity for the receptor, all the antiglucocorticoids tested stabilized the unactivated receptor complexes need to be stressed. Despite no evident structural resemblance to steroids, trifluoroperazine displayed the basic properties of a weak but true antiglucocorticoid (Van Bohemen & Rousseau, 1982). Therefore, the fact that this compound was like steroidal antagonists able to stabilize the 7–8-nm receptor complexes demonstrated that a common pattern of interaction of antiglucocorticoids with the receptor does exist and that dissociation of 7–8-nm receptor complexes constitutes a critical event directly related to receptor activation in vivo. This point needed to be clearly established before trying to understand why antisteroids inhibit GR dissociation.

More intriguing was the observation that some receptor complex dissociation could be observed with native unliganded receptor, even when the rapid HPSEC technique was used. Unliganded receptors lost their binding activity during the chromatographic step and could only be detected by immunoassay. A 6-nm Stokes radius was found, in good agreement with the data reported by Radojcic et al. (1985), who claimed that this form was probably the native untransformed one since it was unable to bind DNA. Our interpretation is quite different, and we propose that this form displaying an intermediate size between the 7–8- and 5.0-nm forms could have resulted from some very rapid artifactual dissociation and/or denaturation during the chromatographic step.

Displacement experiments performed with the GR 49 antireceptor antibody clearly showed a concomitant shift of the steroid-binding activity and of the AC 88 immunoreactivity. It is worth noticing that GR 49, while being able to completely shift the 7.3-nm radioactive peak, could apparently not displace the 8.3-nm peak inconsistently observed in crude rat liver and thymocyte cytosol. However, when 8.3-nm fractions were assayed by Western blot analysis using the same antibody, a receptor band was observed, suggesting that the GR 49 epitope was masked in the 8.3-nm native complexes. The relation of this observation to the structure of the unactivated form of the receptor, its mechanism of dissociation, and the possible role of a RNA molecule in this mechanism will be discussed elsewhere (unpublished observations).

In conclusion, our experimental model, the main features of which were intact cells incubation with various glucocorticoid agonists and antagonists and rapid receptor assay by HPSEC in physiological conditions, gave rise to results in good agreement with some data already obtained by others. For example, RU 486 and dexamethasone mesylate appeared unable to provoke the genomic effects observed with glucocorticoid agonists in intact cells (Becker et al., 1986; Richard-Foy et al., 1987). Moreover, the recent report that RU 486–receptor complexes are not down-regulated in intact cells (Rajpert et al., 1987) affords another argument supporting our data since down-regulation was described as a property linked to receptor dissociation in vivo. All these observations contrast with results obtained in acellular preparations of the antiglucocorticoid receptor complexes in which in vitro activation very probably resulted from artifactual dissociation. Such data reinforce our opinion that progress in the comprehension of the mechanism of glucocorticoid receptor activation imperatively needs to come back to intact cells. Several approaches are conceivable for this purpose, among which resorting to the very important molecular probes constituted by antiglucocorticoid derivatives deserves further development.

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Registry No. RU 486, 84371-65-3; cortexolone, 152-58-9; progesterone, 57-83-0; dexamethasone, 50-02-2; triamcinolone acetonide, 76-25-5; dexamethasone mesylate, 2265-22-7; trifluoroperazine, 117-89-5.

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Structure and Acute-Phase Regulation of the Rat α_2-Macroglobulin Gene†

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ABSTRACT: Seven genomic DNA clones representing the rat α_2-macroglobulin gene were isolated and characterized. The cloned sequence covered the entire gene (48 kilobases) plus 2 kilobases of 3′- and 13.7 kilobases of 5′-flanking sequences. A restriction cleavage map of the gene was produced, and the restriction cleavage pattern of genomic DNA suggested that the α_2-macroglobulin gene is a single-copy gene. A 7.7-kilobase fragment from the 5′-terminal region and a 250 base pair fragment from the 3′-terminal region of the gene were sequenced, and the 3′ end of the gene was mapped. The sequenced 5′-terminal fragment contained 4.5 kilobases of 5′-flanking sequences plus the first three exons and two introns of the gene. Two transcription start sites, a minor and a major site, located 65 nucleotides apart, were defined by primer extension, S1 mapping, and RNaseH experiments. During an acute-phase response, transcription from both sites was induced in the liver, and over 90% of the transcripts originated from the major site. Very high concentrations of α_2-macroglobulin mRNA originating from both start sites were also found in the uterus but not in the liver of pregnant females. A glucocorticoid response element (GRE), a conserved consensus sequence for a potential glucocorticoid receptor DNA binding site, was found by computer search in the promoter-proximal 5′-flanking region of the α_2-macroglobulin gene.

The α_2-macroglobulin (α_2M)[1] is a plasma glycoprotein of high molecular weight (approximate M_r 180 000) which is mainly synthesized in the parenchymal cells of the liver but also in a number of other cell types. During acute and chronic inflammations occurring in response to tissue damage and infections, α_2M concentrations in the plasma are dramatically increased in rats, and α_2M is the major acute-phase protein in rats (Gordon, 1976; Gordon & Koj, 1985; Schreiber, 1987; Lonberg-Holm et al., 1987; Northemann et al., 1985; Hayashida et al., 1985; Gehring et al., 1987). α_2M is a member of the family of rat α-macroglobulins, which includes also α_1-macroglobulin (α_1M) and several species of α_1-inhibitor III (α_1I3). All α-macroglobulins are proteinase inhibitors of high molecular weight. All members of this family contain an internal thiolester bond, which is important for their function, and all are structurally related (Lonberg-Holm et al., 1987; Sottrup-Jensen, 1987; Gehring et al., 1987; Braciak et al., 1988; Gauthier & Ohlsson, 1978; Esnard & Gauthier, 1980; Schaeufele & Koo, 1982). The α-macroglobulins are further structurally related to complement components C3, C4, and C5, and the genes for all family members have evolved from a common ancestor (Sottrup-Jensen et al., 1985; Sottrup-Jensen, 1987). cDNA clones for the major rat α-macroglobulins have been isolated and characterized and were used to establish that the changes of macroglobulin concentrations occurring in the plasma during an acute-phase response are preceded by corresponding changes of their mRNA concentrations in the liver. These changes in mRNA concentrations are caused at least in part by increased transcription of the α_2M gene (Northemann et al., 1985, 198b; Hayashida et al., 1985; Birch & Schreiber, 1986; Gehring et al., 1987). Posttranscriptional alterations in the stability of α_2M mRNA may also participate in the overall changes of α_2M mRNA concentration that occur during an acute-phase response, but they have not yet been demonstrated directly. Transcription of the α_2M gene is increased at least 5-8-fold

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_1M, α_1-macroglobulin; α_1I3, α_1-inhibitor III; AGP, α_1-acid glycoprotein; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); bp, base pair(s); kb, kilobase pair(s); HSF, hepatocyte stimulating factor; IL6, interleukin 6; GRE, glucocorticoid responsive element.

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