Transformation of Calf Uterine Progesterone Receptor: Analysis of the Process When Receptor Is Bound to Progesterone and RU38486†

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ABSTRACT: Effects of different transforming agents were examined on the sedimentation characteristics of calf uterine progesterone receptor (PR) bound to the synthetic progestin [^{3}H]R5020 or the known progesterone antagonist [^{3}H]RU38486 (RU486). [^{3}H]R5020–receptor complexes [progesterone–receptor complexes (PRc)] sedimented as fast migrating 8S moieties in 8–30% linear glycerol gradients containing 0.15 M KCl and 20 mM Na_2MoO_4. Incubation of cytosol containing [^{3}H]PRc at 23 °C for 10–60 min, or at 0 °C with 0.15–0.3 M KCl or 1–10 mM ATP, caused a gradual transformation of PRc to a slow sedimenting 4S form. This 8S to 4S transformation was molybdate sensitive. In contrast, the [^{3}H]-RU486–receptor complex exhibited only the 8S form. Treatment with all three activation agents caused a decrease in the 8S form but no concomitant transformation of the [^{3}H]RU486–receptor complex into the 4S form. PR in the calf uterine cytosol incubated at 23 or at 0 °C with 0.3 M KCl or 10 mM ATP could be subsequently complexed with [^{3}H]R5020 to yield the 4S form of PR. However, the cytosol PR transformed in the absence of any added ligand failed to bind [^{3}H]RU486. Heat treatment of both [^{3}H]R5020– and [^{3}H]RU486–receptor complexes caused an increase in DNA–cellulose binding, although the extent of this binding was lower when RU486 was bound to receptors. An aqueous two-phase partitioning analysis revealed a significant change in the surface properties of PR following both binding to ligand and subsequent transformation. The partition coefficient (K_{obsd}) of the heat-transformed [^{3}H]R5020–receptor complex increased about 5-fold over that observed with PR at 0 °C. Under the same conditions, the heat-treated [^{3}H]RU486–receptor complex showed only a small increase over the value observed with unheated samples containing RU486-bound receptors. Our results demonstrate for the first time that calf uterine PR can be transformed in vitro by heat, salt, and ATP, both in the absence and in the presence of the progesterone agonist R5020. Transformation of the unoccupied calf uterine PR appears to involve conformational changes that allow its subsequent binding to the agonist R5020, but not to the antagonist RU486. Conversely, transformation of [^{3}H]RU486-bound PR with known transforming agents appears to be prevented along with those changes that are required for the 8S to 4S transformation. However, the antigestational actions of RU486 reported in the literature may not be solely related to in vitro DNA binding and the impaired ability of RU486-bound PR to undergo the 8S to 4S transition.

Steroid hormones are known to interact with their respective specific intracellular receptors. The exact cellular compartment where the initial interaction between a steroid hormone and its receptor takes place has been the subject of numerous investigations. Results of many recent studies have indicated that unoccupied receptors for estrogen, progesterone, and glucocorticosteroid may reside in the nucleus of target cells (King & Greene, 1984; Gasc et al., 1984; Welshons et al., 1984, 1985; Shull et al., 1985). The receptor that is generally recovered in the cytosol fraction of a homogenate may, therefore, represent a receptor that is loosely associated with the nucleus, and its binding with the hormone leads to a tighter association of the steroid–receptor complex to the nuclear sites. Although it is widely believed that steroid hormones bring about their effects by altering the expression of steroid-responsive genes, the exact sequence of events leading to such a cellular response is not well understood.

Under physiological conditions, upon binding of a steroid hormone to its receptor, the steroid–receptor complex is thought to undergo a temperature-dependent process called "activation" or "transformation"[1] which leads to its increased binding to certain as yet undefined nuclear chromatin sites (Milgrom, 1981). In cell-free systems, unoccupied steroid receptors are extracted in the cytosol fraction where they can be complexed with steroids to form "nontransformed" or "nonactivated" steroid–receptor complexes which sediment as 8–9S molecules and exhibit poor affinity for isolated target cell nuclei and certain polyanions (Milgrom, 1981). Incubation of these complexes at 23–27 and at 0–4 °C with ATP or KCl transforms the 8–9S receptor into a slow migrating 4S form which displays an affinity for isolated nuclei, DNA–cellulose, and ATP–Sepharose (Milgrom, 1981; Moudgil et al., 1981, 1985).

In this report, we have studied the transformation of calf uterine progesterone receptor (PR)[2] under a variety of experimental conditions. The transformation of PR bound to the known progesterone antagonist RU486 was studied in

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[1] The term transformation refers to an in vitro alteration of the cytosol receptor from its 8S form to a form that sediments as 4 S. The same term has also been used to represent the in vitro alteration of the cytosol receptor to the form that binds to isolated nuclei, DNA–cellulose, or ATP–Sepharose.

[2] Abbreviations: EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; PR, progesterone receptor; PRc, progesterone–receptor complex(es); ATPP, aqueous two-phase partitioning; R5020, 17,21-dimethylpregna-4,9(10)-diene-3,20-dione; RU38486, 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-(prop-1-ynyl)-estra-4,9-dien-3-one; Tris, tris(hydroxymethyl)aminomethane; HAP–PR, hydroxylapatite–progesterone receptor; PEG, poly(ethylene glycol); GR, glucocorticoid receptor; MMTV, mouse mammary tumor virus.

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parallel with the agonist R5020 in order to obtain insight into the mode of action of this recently synthesized antihormone.

Materials and Methods

Chemicals. All reagents were of analytical grade and were prepared in deionized water. [^{3}H]Progesterone (40–50 Ci/mmol), [^{3}H]R5020 (70–87 Ci/mmol), and R5020 were purchased from New England Nuclear (Boston, MA). [^{3}H]-RU38486 (30–35 Ci/mmol) and radioinert RU38486 were obtained as a gift from Roussel Uclaf (Romainville, France). All procedures were carried out at 0–4 °C unless indicated otherwise. ATP, progesterone, cortisol, Na_2MoO_4, glycerol, EDTA, monothioglycerol, charcoal, and calf thymus DNA were all purchased from Sigma Chemical Co. (St. Louis, MO). DNA-grade hydroxylapatite and cellulose powder were from Bio-Rad Laboratories (Richmond, CA); Ultrapure Tris was obtained from Schwarz/Mann Co. (Cambridge, MA). Dextran T-70 was from Pharmacia (Uppsala, Sweden). Glucose oxidase and horseradish peroxidase were obtained from Boehringer-Mannheim (Indianapolis, IN). ATPP polymer solutions were received as gift from Dr. Jeff Hansen (Madison, WI).

Buffers. The following buffers were used: buffer A—20 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, and 8% glycerol, pH 7.5 at 23 °C; buffer B—20 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, and 20% glycerol, pH 7.5 at 23 °C; buffer C—20 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, 1 M KCl, and 20% glycerol, pH 7.5 at 23 °C; buffer D—20 mM Tris-HCl, 1 mM EDTA, and 12 mM monothioglycerol, pH 7.5 at 23 °C.

Preparation of Calf Uterine Cytosol. Calf uteri were obtained from a local meat processing company. The calves were sacrificed approximately 10 min before the uteri were removed. After removal, the tissue was packed in ice and transported to the lab. The tissue was excised, rinsed in cold 0.9% NaCl, quick-frozen in precooled (−70 °C) ethanol, and stored at −70 °C until used. To prepare cytosol, the tissue was minced, rinsed with cold 0.9% NaCl, and homogenized in 4 volumes (v/w) of buffer A with four 5–10-s bursts using a tissumizer (Tekmar, Model SDT). PMSF was added to the homogenate to a final concentration of 0.3 mM just after the first homogenization burst. The homogenate was centrifuged for 10 min at 10000*g*. The supernatant was collected and centrifuged 1 h at 150000*g*. The resultant cytosol was collected by aspirating with a Pasteur pipet between the lipid layer on the surface and pellet. The cytosol was incubated 10 min with 3 μM cortisol to saturate corticosteroid binding sites.

Formation of Steroid–Receptor Complex. Cytosol was incubated 2–4 h at 0 °C with 20 nM [^{3}H]progesterone, [^{3}H]R5020, or [^{3}H]RU486. In order to determine specific binding, aliquots of cytosol were incubated in duplicate with 2 μM radioinert steroids for 10 min prior to incubation with ^{3}H-steroids.

Transformation of PRc. Transformation of PRc was accomplished by incubating aliquots of cytosol with 0.1–0.3 M KCl for 30 min or 1–10 mM ATP for 30–120 min or by exposing cytosol to 23 °C for 30–60 min. Portions of cytosol PRc or unbound receptors were thermally transformed by incubating cytosol at 23 °C for 10–120 min. A control for transformation was employed in each set of experiments, which consisted of cytosol PRc that remained at 0–4 °C with or without 20 mM Na_2MoO_4. At the end of each incubation period, except where noted, Na_2MoO_4 was added to a final concentration of 20 mM to block further transformation.

Sedimentation Rate Analysis. Continuous 8–30% linear glycerol gradients (4.4 mL) in buffer B containing 0.01 M KCl were prepared by using a Beckman density gradient former (Beckman, Palo Alto, CA). After the gradients were precooled (4 °C) for 1 h, aliquots (0.2 mL) containing PRc were layered onto the gradients. In some cases, PRc samples were incubated, just prior to being layered onto the gradients, for 5–10 min with a pellet formed by sedimenting an equal volume of charcoal suspension (0.5% activated charcoal and 0.05% dextran in buffer F). Gradients were then centrifuged at 270000*g* in a Sorvall TV-865 vertical rotor (Ivan Sorvall, Inc., Norwalk, CT) for 2 h and 15 min. At the end of the run, the gradient tubes were pierced at the bottom, and 21–23 0.2-mL fractions were collected into scintillation vials.

DNA Binding Assay. DNA–cellulose was prepared according to the method of Alberts and Herrick (1971). Calf thymus DNA (type II) was linked to Cellex-410. Our preparations contained 1.5–2 mg of DNA/g of packed DNA–cellulose as quantitated by the procedure of Burton (1956). For performing batch assays, pellets prepared from aliquots of DNA–cellulose suspension (10 mg/mL containing 15–300 μg of DNA) were incubated 30 min at 0 °C with 200 μL of PRc preparation. The suspensions were centrifuged for 5 min at 1000*g* to obtain pellets. Supernatant was removed, and the pellets were washed 3 times with buffer B. PRc bound to DNA–cellulose were recovered by incubating pellets with 1 mL of buffer C for 30 min at 0 °C. After centrifugation for 5 min at 1000*g*, the supernatants were collected and transferred into scintillation vials. For column chromatography, 1-mL columns of DNA–cellulose were prepared and equilibrated with 5 volumes of buffer B. Aliquots (1 mL) containing PRc were layered onto the columns. The adsorbed PRc were eluted with buffers B and C. Portions (0.5 mL) of these fractions were used for measurement of radioactivity.

Steroid Binding Measurements. For quantitation of steroid–receptor complexes, portions of cytosol containing PRc were incubated for 5–10 min at 0 °C with an equal volume of charcoal suspension or with a pellet formed from centrifuging an equal volume (v/v) of dextran-coated charcoal suspension (0.5% activated charcoal and 0.05% dextran in buffer F). Mixtures were centrifuged for 5 min at 1000*g*, and the supernatants were removed and placed in scintillation vials for measurement of radioactivity.

Aqueous Two-Phase Partition Analysis. Aqueous two-phase partitioning (ATPP) was utilized in conjunction with the hydroxylapatite–progesterone receptor (HAP–PR) assay according to the methods of Hansen and Gorski (1985). In experiments performed on unoccupied PR, aliquots of cytosol were partitioned immediately after cytosol was prepared as described below. In experiments involving liganded receptor, cytosol was incubated with [^{3}H]R5020 or [^{3}H]RU486 for 2 h prior to partitioning. For each condition, parallel analysis with excess nonradioactive steroid was performed in order to determine the amount of nonspecific binding.

Stock solutions of polymer were stored as 28.82% poly(ethylene glycol) (PEG, M_r 8000) and 24.325% dextran (M_r 461 000 and 510 000) in TED buffer (10 mM Tris-HCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol). The phase system, composed of 9% (w/w) dextran and PEG, was formed by combining stock solutions and buffer D in the proper proportions. The mixture was stirred until turbid. Aliquots (0.9 mL) of this polymer phase system were added to portions (0.6 mL) of PRc or PR preparations. The mixtures were vortexed for 10 s and then centrifuged for 5 min at 1000*g* to form the characteristic biphasic system. For determination of the steroid receptor distribution in the phases, the HAP assay was employed. Small aliquots (0.2 mL) from each phase were re-

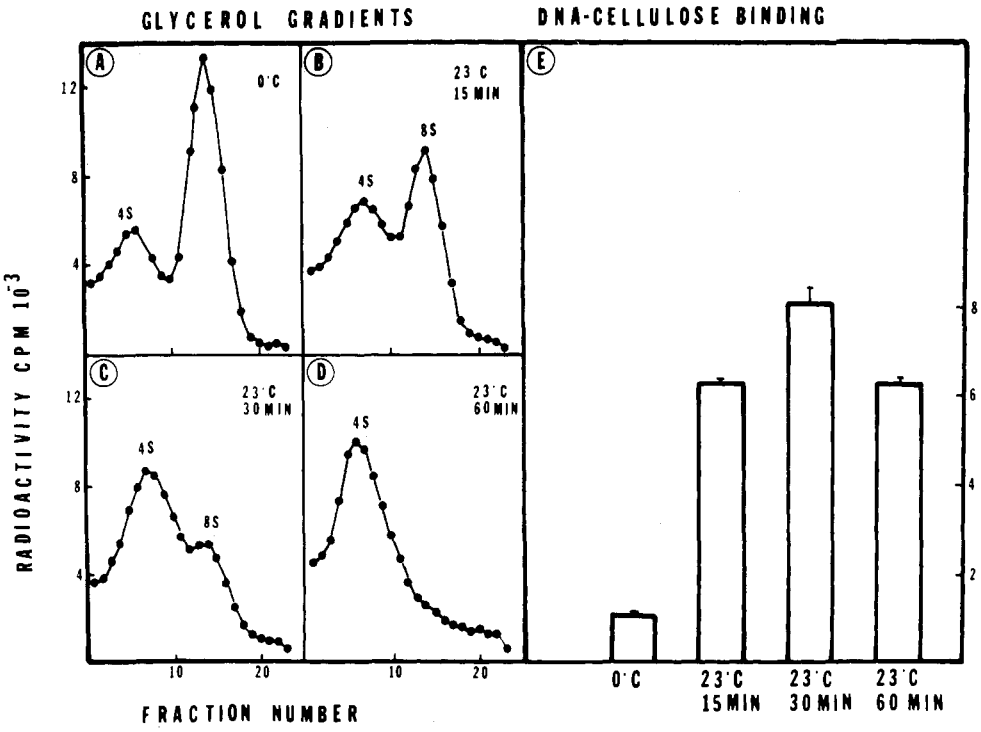

FIGURE 1: Effect of heat on the transformation of the [^{3}H]R5020–receptor complex. Aliquots of cytosol containing [^{3}H]R5020–receptor complexes were incubated at 23 °C for 0, 15, 30, and 60 min. At the end of each heat treatment period, sodium molybdate was added to a 20 mM concentration. Aliquots (0.2 mL) were analyzed on 8–30% linear glycerol gradients containing 0.15 M KCl and 20 mM Na_2MoO_4. Internal markers used for reference were glucose oxidase (7.9 S) and peroxidase (3.6 S). The gradients were centrifuged at 270000*g* for 2 h at 4 °C in a Sorvall vertical rotor. Fractions (0.20 mL) were obtained by piercing the bottom of the tubes. Duplicate 0.2-mL samples were also used to determine the extent of DNA–cellulose binding. Panels A–D represent gradient analyses of receptor heat treated for 0, 15, 30, and 60 min, respectively. Panel E shows the extent of DNA binding at the same conditions.

moved and placed in separate tubes. Unoccupied receptor was labeled with 20 nM [^{3}H]R5020 or [^{3}H]RU486 for 2 h at 0 °C. The HAP assay described by Williams and Gorski (1974) was performed on all samples. Partition coefficients were determined by dividing the specific counts per minute in the upper phase by the specific counts per minute in the lower phase.

RESULTS

A majority (80%) of the PRc from calf uterine cytosol, prepared in salt-free buffer, sedimented close to the 7–9S region (Figure 1A). For convenience, we shall henceforth refer to this as the 8S PR form. The 8S form represents the nonactivated PR (Nishigori & Toft, 1980; Wolfson et al., 1980; Moudgil, 1985). Incubation of cytosol at 23 °C for 15–60 min caused an increase in the amount of a smaller [^{3}H]PRc form which sedimented slightly ahead of the 3.6S marker (Figure 1B–D). This form will be referred to as the 4S PR, and it has previously been shown to represent the activated or transformed receptor (Nishigori & Toft, 1980; Moudgil, 1985). The heat treatment increased the proportion of the 4S PR from 20% to nearly 100% of the receptor population. The gradual conversion of the entire receptor population into the 4S form was also accompanied by a loss of between 20% and 30% progesterone–receptor complexes (not shown). The 8S to 4S PR transition following heat treatment was accompanied by a parallel increase in the ability of PRc to bind DNA–cellulose (Figure 1E). The results illustrated in Figure 1 demonstrate that PR from calf uterine cytosol undergoes the process of transformation exhibited by other steroid receptors under a variety of experimental conditions (Moudgil, 1985).

Figure 2 illustrates the effect of elevated temperature on the sedimentation rate and DNA binding ability of PR complexed with [^{3}H]R5020 or [^{3}H]RU486. While a 60-min incubation of uterine cytosol containing [^{3}H]R5020–receptor complexes resulted in the appearance of 82% of the complexes in the preparation as the 4S form (Figure 2A,B), the quantifiable population of [^{3}H]RU486–receptor complexes remained in the 8S form (Figure 2C). There was a decrease (53%) in the number of 8S [^{3}H]RU486–receptor complexes, but no concomitant increase in, nor the appearance of, the 4S receptor peak was observed (Figure 2D). This observation does not exclude the possibility that the receptor was more rapidly inactivated or degraded in the presence of RU486 than in the presence of the agonist. When portions of the above samples were used to determine the extent of DNA–cellulose binding under the same conditions, the results were somewhat different. Nontransformed PR complexes exhibited only a limited affinity for DNA–cellulose, irrespective of the nature of the ligand bound to the receptor. Following an incubation of cytosol at 23 °C for 60 min, there was a 10–15-fold increase in the DNA–cellulose binding ability of both the [^{3}H]R5020- and [^{3}H]RU486-bound PR complexes, although the latter achieved only 70–80% of the level of binding observed with [^{3}H]R5020. It appears that the [^{3}H]RU486–receptor complex can be activated, and DNA binding measured during a 30-min assay, but the 4S activated complex is unstable and dissociates during the 2 h, 15 min ultracentrifugation assay at 270000*g*.

In order to determine the influence of thermal transformation on the stability of steroid–receptor complexes, the number of complexes remaining after a 23 °C incubation was determined by a charcoal adsorption assay. Figure 3 illustrates the effect of the time at 23 °C on the population of the specifically bound progesterone- and RU486–receptor complexes. The rate of loss of PRc was greater than for RU486–receptor complexes. The greatest difference in the binding of these two ligands appeared after 1 h of heat treatment when the total

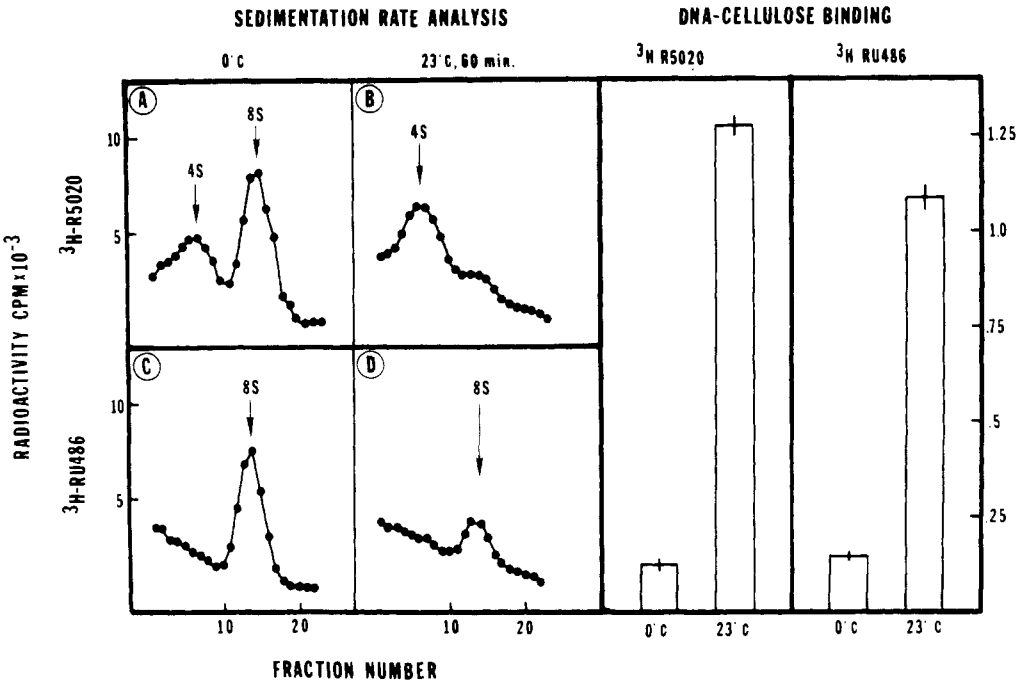

FIGURE 2: 8S to 4S transformation and DNA binding of PR bound to [^{3}H]R5020 or [^{3}H]RU486. Cytosol was prepared in buffer A plus 3 μM cortisol and incubated for 2 h with 20 nM [^{3}H]R5020 or [^{3}H]RU486. Aliquots of the above receptor preparation were incubated at 23 °C for 60 min or retained at 0 °C to serve as controls. Sodium molybdate was added to 20 mM concentration. The samples were charcoal treated, and 0.2-mL samples for each condition were analyzed on 8–30% linear glycerol gradients. Duplicate samples (0.2 mL) were also assayed to determine the extent of DNA–cellulose binding. (A) [^{3}H]R5020–receptor complex, 0 °C; (B) A+ 23 °C, 60 min; (C) [^{3}H]RU486–receptor complex, 0 °C; (D) C+ 23 °C, 60 min. The right-hand panels show the extent of DNA–cellulose binding of cytosol aliquots incubated with [^{3}H]R5020 and [^{3}H]RU486.

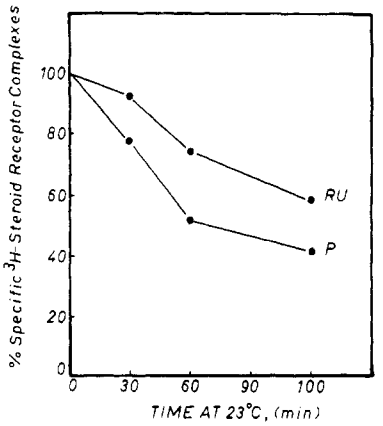

FIGURE 3: Quantitation of the specific steroid–receptor complexes after heat treatment. Cytosol was prepared as described earlier and contained 3 μM cortisol. Aliquots of cytosol were incubated for 2 h at 4 °C with 20 nM [^{3}H]progesterone and [^{3}H]RU486. Parallel samples were incubated with 100-fold radioinert steroids for 10 min prior to incubation with labeled steroids. Aliquots of cytosol were then removed from ice and incubated at 23 °C for 0, 30, 60, and 120 min. All samples were then treated with charcoal to determine the amount of specifically bound ^{3}H-steroid. The results show the amount of specific binding that remained at a given time of exposure of the complexes to 23 °C. 100% binding represents the number of specific steroid–receptor complexes present prior to the incubation at 23 °C.

number of RU486–receptor complexes had only declined by 25% but the amount of progesterone–receptor complexes had dropped by 50%. In light of this observation, it appears that PR is degraded more rapidly during the ultracentrifugation run when it is bound to RU486.

The results of Figure 2 raised the possibility that nontransformed receptor might have contributed to the observed DNA–cellulose binding of heat-treated [^{3}H]RU486–receptor complexes. As demonstrated in Figure 4, the nontransformed PR bound to either of the steroids exhibited no retention on the DNA–cellulose column, and the complexes from the flow-through sedimented in the 8S region. Heat treatment of the cytosol containing both types of PR complexes increased the extent of DNA–cellulose binding. When the resin-bound [^{3}H]R5020– and [^{3}H]RU486–receptor complexes were extracted and analyzed on glycerol gradients, both types of the complexes migrated as slow sedimenting moieties in the 3–4S region. The peaks of radioactivity due to steroid–receptor complexes extracted from DNA–cellulose were not sharp, presumably due to the instability and labile nature of DNA–cellulose-extracted receptor.

In order to determine whether or not the effect of elevated temperature on the rate of sedimentation of PR represented a general phenomenon, the influence of other agents on the receptor transformation was also tested. Figure 5 demonstrates that when cytosol complexed with [^{3}H]progesterone or [^{3}H]R5020 was incubated at 0 °C with 10 mM ATP (pH 8), the 8S PR was converted to the slow migrating 4S form. The extent of this receptor transformation appeared to be greater when the ligand was [^{3}H]progesterone. The ATP treatment resulted in the transformation of 75% of the [^{3}H]progesterone–receptor complexes to the 4S form whereas only 57% of the [^{3}H]R5020–receptor complexes sedimented as 4S moieties under these conditions. However, when the cytosol was complexed with [^{3}H]RU486, ATP treatment of the preparation resulted in a decrease in the size of the 8S peak, but a distinct 4S peak seen with the transformed receptor liganded to the other two steroids was not observed. A similar comparative study revealed comparable results when the transformation was induced by a 0 °C incubation of the cytosol preparation with 0.15–0.3 M KCl (Figure 6). As expected, an increase in ionic strength (0.15–0.3 M KCl) caused a transformation of the 8S [^{3}H]R5020–receptor complex with an appearance of 22–70% of the complexes in the 4S receptor form. The rate of sedimentation of [^{3}H]RU486–receptor complexes remained unaltered under the same conditions; the

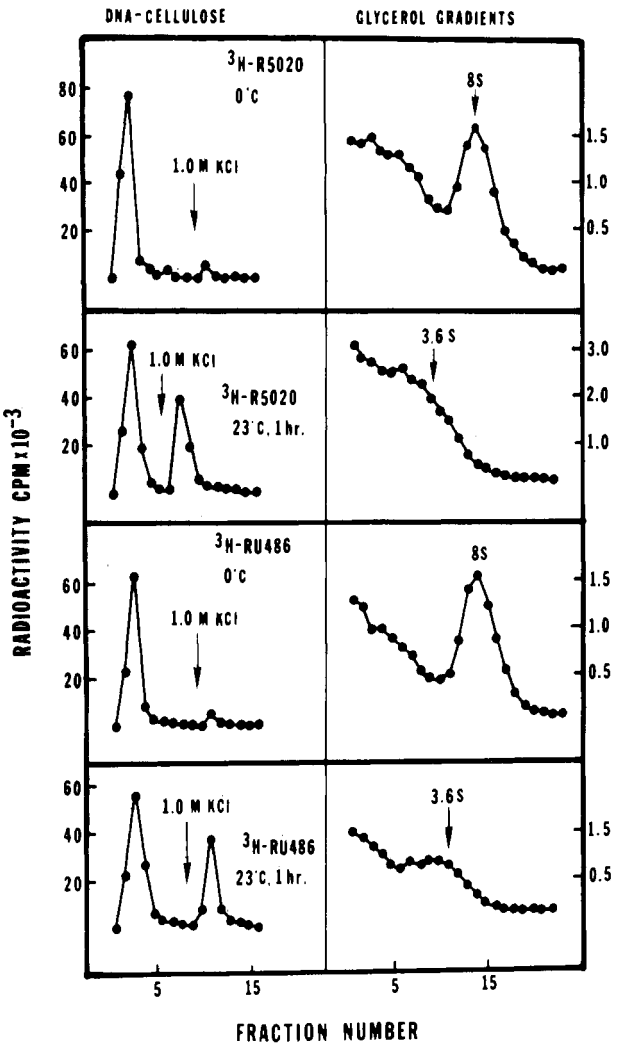

FIGURE 4: Sedimentation rate analysis of PR extracted from DNA–cellulose. Cytosol was prepared in buffer A plus 3 μM cortisol and incubated 2 h with 20 nM [^{3}H]R5020 or [^{3}H]RU486. Aliquots from the above preparation were incubated for 60 min at 23 °C or kept at 0 °C as controls. Portions (1 mL) of heat-treated and control cytosol were chromatographed through 1-mL DNA–cellulose columns. Ten 1-mL fractions were collected each with buffers B and C. The left-hand panels show the DNA–cellulose binding of R5020- and RU486-bound PR in the control and heat-treated samples. Portions (0.2 mL) of peak fractions collected with buffer B (from control samples) and with buffer C (heat-treated samples) were analyzed on 8–30% linear glycerol gradients.

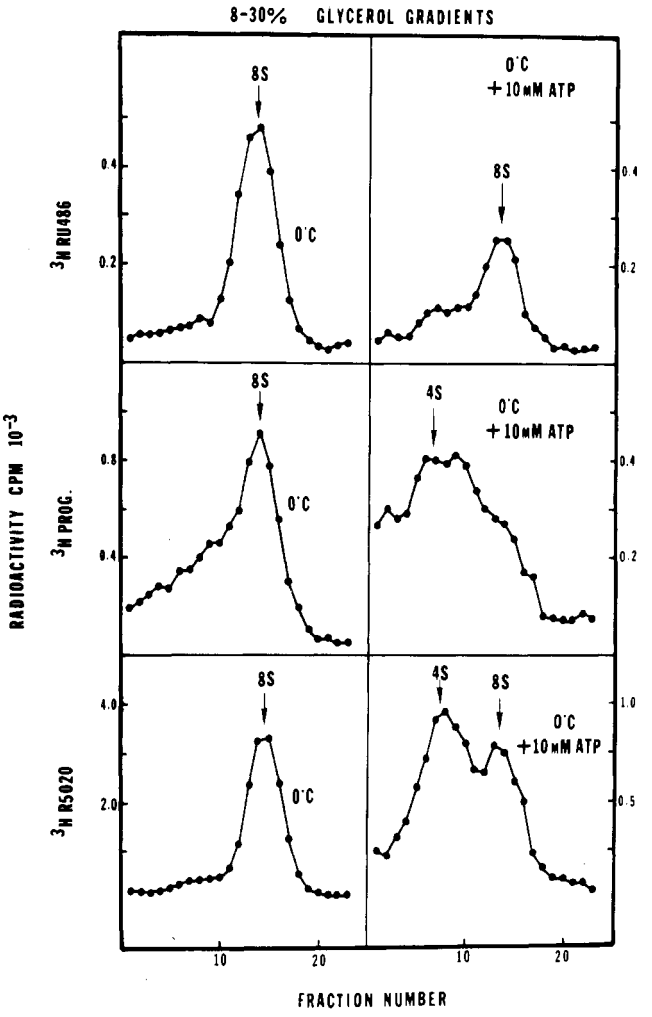

FIGURE 5: Effect of ATP on the sedimentation rate of CU–PR. Cytosol was prepared in buffer A plus 3 μM cortisol and incubated for 2 h with 20 nM [^{3}H]RU486, [^{3}H]progesterone, or [^{3}H]R5020 to form steroid–receptor complexes. Aliquots of the above were incubated in the presence and absence of 10 mM ATP for 2 h at 0 °C. Sodium molybdate was then added to 20 mM concentration. The samples were charcoal treated, and 0.2-mL samples were analyzed on 8–30% linear glycerol gradients.

8S peak remained intact, and no peak of radioactivity in the 4S region was evident.

Figure 7 shows a detailed analysis of the effects of all three transformation-inducing agents on the 8S [^{3}H]R5020 and [^{3}H]RU486 binding components of the calf uterine cytosol. It is clear that the ability of the 8S nontransformed progesterone receptor to transform into a slow migrating 4S form is reduced when its steroid binding site(s) is (are) occupied by the antiprogesterone [^{3}H]RU486. At conditions (23 °C, KCl and ATP) where 50% transformation of the 8S [^{3}H]-R5020–receptor complexes was accomplished, only 15–18% reduction in the 8S [^{3}H]RU486–receptor complexes was observed. The lower degree of transformation seen with the heat-treated [^{3}H]RU486–receptor complex (Figure 7, left) may be due either to a lower rate of transformation of the receptor or to a dissociation of the steroid from an activated antagonist-bound PR.

Inclusion of 10–20 mM Na_2MoO_4 in the homogenization buffer or in freshly prepared cytosol is known to stabilize avian progesterone receptor in its nontransformed, non-DNA/ATP binding 8S form (Nishigori & Toft, 1980; Moudgil et al., 1981). Although molybdate is a general inhibitor of receptor transformation, the degree and extent to which the molybdate presence blocks the effects of various transformation-inducing agents vary (Toft et al., 1980). In this study, we have examined the effect of the addition of 20 mM Na_2MoO_4 on the heat-, KCl-, and ATP-dependent transformation of [^{3}H]-R5020–receptor complexes. Results shown in Figure 8 demonstrate that all three agents (left panel) cause 8S to 4S conversion of cytosolic [^{3}H]R5020–receptor complexes. However, when portions of the cytosol preparation were treated with 20 mM Na_2MoO_4 prior to heat treatment or addition of ATP or KCl, the ability of all three agents to transform the receptor was blocked. None was able to fully achieve the 8S to 4S transformation in the presence of Na_2MoO_4. This observation suggests that PR from calf uterus exhibits properties similar to those shown by PR from avian target tissues. In light of this evidence, we have included 20 mM Na_2MoO_4 in our gradient buffers to reduce further transformation during the ultracentrifugation of gradients.

To examine the role of steroid in the transformation of 8S PR to the 4S form, freshly prepared cytosol was incubated at 0 and 23 °C for 1 h prior to the addition of radiolabeled

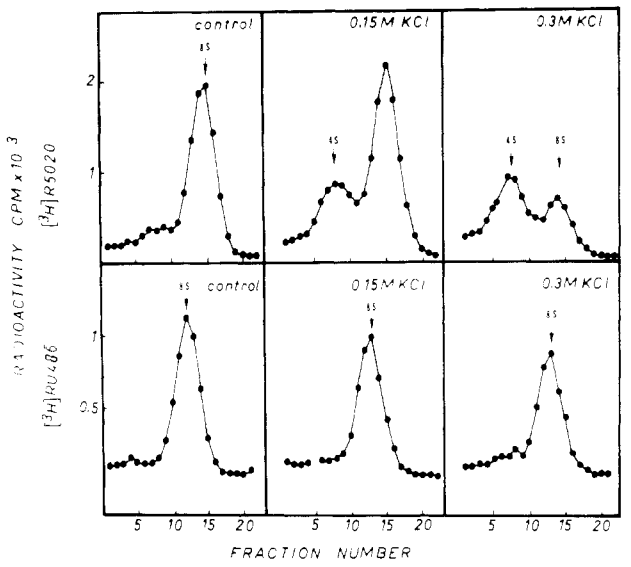

FIGURE 6: Effect of salt on the sedimentation profiles of PR bound to [^{3}H]R5020 and [^{3}H]RU486. Cytosol was incubated with 20 nM [^{3}H]R5020 or [^{3}H]RU486. Aliquots of the above were incubated without salt (control) and with 0.15 or 0.3 M KCl at 0 °C for 30 min. Sodium molybdate was added to a 20 mM concentration, and 0.2-mL samples were layered on 8–30% linear glycerol gradients.

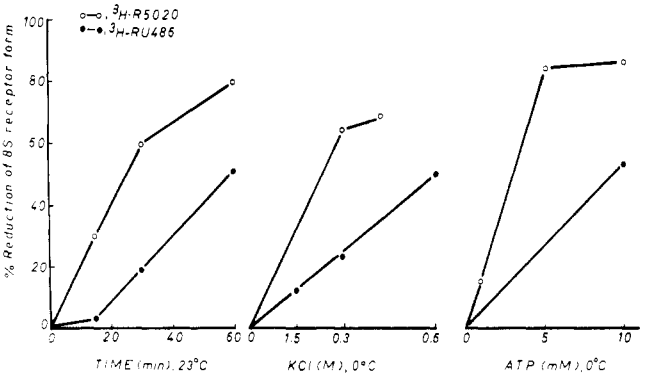

FIGURE 7: Effect of heat, salt, and ATP on the 8S form of the calf uterine progesterone receptor. Cytosol aliquots were mixed with 3 μM cortisol and incubated with 20 nM [^{3}H]R5020 (○) or [^{3}H]RU486 (●) for 2 h. Portions of the above were then incubated at 23 °C for 0–60 min (left panel), for 40 min at 0 °C with 0–0.6 M KCl (center panel), or for 1 h at 0 °C with 0–10 mM ATP. Samples (0.2 mL) from each group were layered over 8–30% linear glycerol gradients. The fractions (0.21 mL) were collected by piercing the bottom of the tubes. The area under the 8S peak was calculated for each gradient. The figure shows the percent reduction of the area under the 8S peak as a function of time of incubation at 23 °C and KCl and ATP concentration.

steroids. Subsequently, it was divided into two portions, and aliquots of each were labeled separately with 20 nM [^{3}H]-R5020 or [^{3}H]RU486. After 2 h at 0 °C, samples were used for sedimentation rate analysis. Figure 9 (left panel) illustrates that PR can be transformed in its ligand-free state and that the [^{3}H]R5020–receptor complexes formed subsequently sediment in the 4S region. The transformed unliganded PR, however, showed no affinity for [^{3}H]RU486 (right panel). Only a small portion of the total population of pretransformed PR labeled with [^{3}H]RU486 migrated in the 8S region with no detectable peak of radioactivity in the 4S region. Results illustrated in Figure 9 show that unliganded receptor can be transformed to the 4S form which retains the ability to bind [^{3}H]R5020 but not [^{3}H]RU486. It is also possible that in the cytosol incubated at 23 °C in the absence of steroid, [^{3}H]-R5020 associated only with the 8S PR, but the [^{3}H]R5020–receptor complexes lost the steroid during ultracentrifugation,

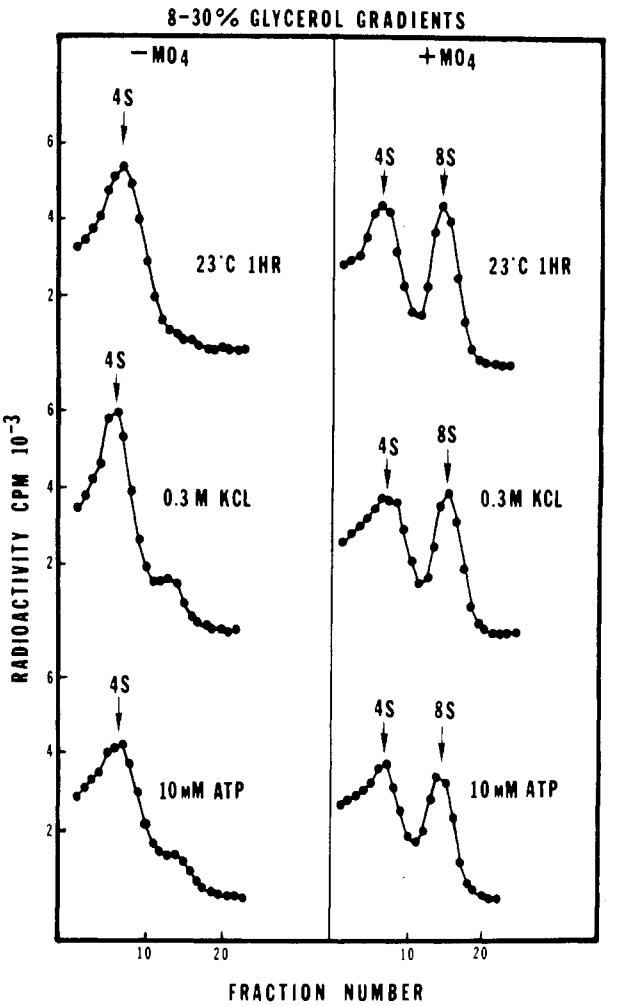

FIGURE 8: Effect of molybdate on the 8S to 4S transformation of the [^{3}H]R5020–receptor complex. [^{3}H]R5020–receptor complexes were transformed by heat (1 h at 23 °C), salt (30 min at 0 °C with 0.3 M KCl), or ATP (30 min, 0 °C, 10 mM ATP) in the presence or absence of 20 mM molybdate. Molybdate was then added to 20 mM concentration to those samples that were not incubated previously with molybdate. Subsequently 0.2-mL samples were analyzed over 8–30% linear glycerol gradients.

and the latter (steroid) was recovered in the 4S region. It is, therefore, difficult to establish, in this instance, whether receptor transformation occurred in the absence of hormone. However, in vitro transformation of unliganded progesterone receptor has been reported in the literature and will be considered under Discussion. Finally, the results presented in Figure 9 and in Figures 1 and 5 do not exclude the possibility that the data represent some receptor degradation, dissociation of the receptor–ligand complex, or both of the above possibilities.

Andreasen (1981) initially employed the aqueous two-phase partitioning (ATPP) technique to study a number of characteristics of the transformation of rat liver glucocorticoid receptor (GR). More recently, Hansen and Gorski (1985) described the effectiveness of the ATPP technique for determining conformational and electrostatic properties of unoccupied and liganded estrogen receptors. Since ATPP is governed by thermodynamic principles, and is independently sensitive to both conformational and electrostatic properties of proteins, it could be used as an alternative or an adjunct to the widely used hydrodynamic techniques which work optimally under nonequilibrium conditions. Results of Figure 10 demonstrate that the ATPP technique can discriminate between ligand-free and ligand-bound nontransformed and

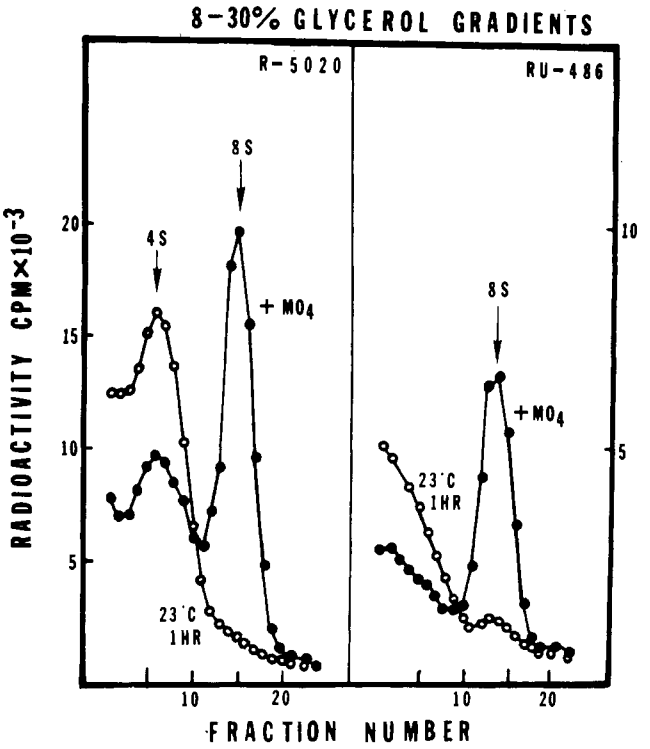

FIGURE 9: Transformation in the absence of ligand. Cytosol was prepared in buffer A plus 3 μM cortisol. Portions of cytosol were incubated 60 min at 23 °C (○) or kept at 0 °C [(●) controls]. Molybdate was then added to 20 nM concentration to all the samples. Heat-treated cytosol and control cytosol were then divided and incubated with either 20 nM [^{3}H]R5020 or [^{3}H]RU486 for 2 h. Samples (0.2 mL) for each condition were then analyzed on 8–30% linear glycerol gradients.

transformed progesterone receptor from calf uterine cytosol as previously reported in the case of estradiol receptor (Hansen & Gorski, 1985). The partition coefficient (K_{obsd}) of the heat-transformed [^{3}H]R5020–receptor complex increased about 5-fold over that seen with the nontransformed [^{3}H]-R5020–receptor complex that remained at 0 °C. Under the same conditions, the heat-treated [^{3}H]RU486-bound progesterone receptor showed only a small increase over the value observed with unheated samples, indicating that it was not transformed (Figure 10, left). The effect of heat (23 °C) on the rate of transformation of RU486– and R5020–receptor complexes was examined over a period of 60 min (Figure 10, middle); [^{3}H]R5020–receptor complexes showed a 2–3-fold higher K_{obsd} value over that seen with [^{3}H]RU486–receptor complexes. These data provide additional evidence that there are molecular differences between unliganded, liganded nontransformed, and heat-transformed PR. Since the lower of the polymer phases is more hydrophilic than the upper, the increased K_{obsd} value in the case of the transformed receptor suggests that transformation renders the progesterone receptor more hydrophobic. A relatively small increase in the K_{obsd} of [^{3}H]RU486-bound PR suggests that different conformational changes and/or charge distribution on the surface of the receptor are involved in the heat transformation of R5020- and RU486-liganded PR. Transformation of PR bound to either of the ligands resulted in the loss of a number of complexes, the loss being greater with R5020-bound PR (Figure 10, right). This observation is consistent with an earlier suggestion that inactivation of steroid binding ability and transformation of steroid–receptor complexes are related phenomena (McBlain et al., 1981).

Discussion

Transformation of steroid receptors is generally studied by measuring the nuclear uptake, DNA–cellulose, ATP–Sepharose, or phosphocellulose binding (Milgrom et al., 1973; Moudgil & Toft, 1975; Moudgil & John, 1980; Milgrom, 1981). When applied simultaneously, these methods yield proportional but nonidentical results (Yang et al., 1982). In this study, we have investigated various parameters of the transformation of calf uterine cytosol PR employing three different end-point assays (DNA–cellulose, aqueous partitioning, and ultracentrifugation analysis) for the measurement of receptor transformation. The main technique employed, however, was the size separation method. The untransformed and transformed PR species were separated on the basis of their rate of sedimentation in glycerol gradients. The latter appeared to yield a better separation of the radioactivity peaks when compared with conventionally used sucrose gradients.

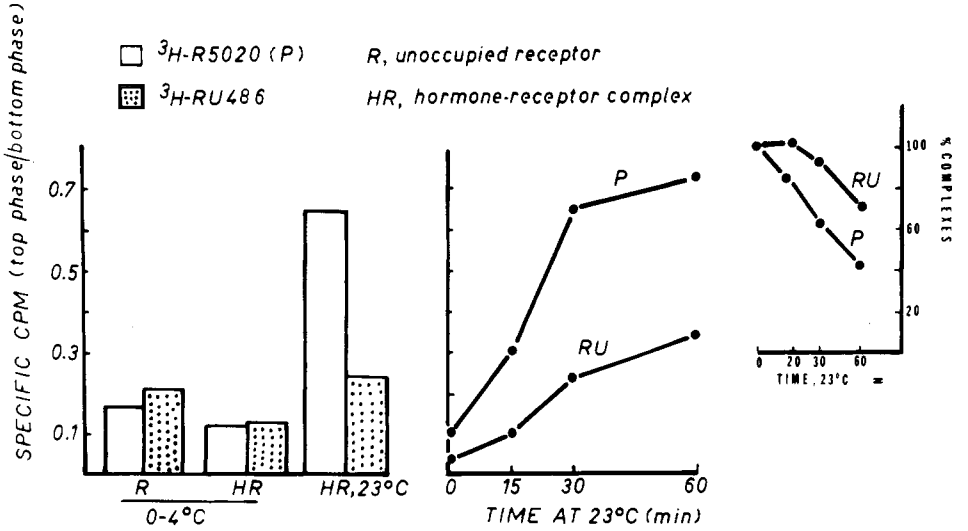

FIGURE 10: Aqueous two-phase partitioning of calf uterine PR. For experiments designed to determine the partition coefficient of unbound receptor, aliquots of cytosol were partitioned prior to incubating with [^{3}H]R5020 or [^{3}H]RU486. For the analysis of liganded receptor, cytosol was first incubated with 20 nM [^{3}H]R5020 or [^{3}H]RU486 prior to partitioning. The complexes were then incubated for 0–60 min at 23 °C and subjected to partitioning. The left panel shows the partition coefficients of unoccupied PR receptor, [^{3}H]R5020, or [^{3}H]RU486-bound receptors, and PR heat transformed at 23 °C for 1 h. The middle panel shows the calculated partition coefficients for PR bound to R5020 and RU486 as a function of time of 23 °C incubation. The panel on the right shows the number of PR complexes remaining after heat treatment for up to 60 min.

There are advantages to the use of the size separation method for studying receptor transformation. While other methods of measurement allow only a partial estimation of the number of transformed complexes, a total transition of receptor from its nontransformed to transformed state can be observed by this method. In addition, analysis of receptor can be performed without removal of the transforming agents as the gradient solutions were supplemented with 20 mM Na_2MoO_4 to block further activation and impart stability to the receptor. Using this method, we have compared the sedimentation characteristics of PR bound to the synthetic progestin agonist R5020 with the newly synthesized progestin antagonist RU486. The rate of sedimentation of transformed and untransformed species observed in this study agrees well with the data reported by Theofan and Notides (1984).

Under physiological conditions, transformation of steroid receptors is known to be a hormone-dependent process (Munck & Foley, 1979; Markovic & Litwack, 1980). Buller et al. (1972) had previously shown that nuclear uptake of PR was dependent upon binding of the hormone. In cell-free conditions, transformation of rat liver GR could only be seen when the receptor was occupied with the ligand. However, transformation of chick oviduct PR can be accomplished in vitro in the absence of hormone (Moudgil et al., 1985), although heat transformation of chick PR was noted to be significantly accelerated in the presence of the hormone. Conflicting data exist on the hormone dependency of in vitro transformation of glucocorticoid receptor. Transformation of rat liver glucocorticoid receptor under cell-free conditions has been shown to exhibit an absolute hormone requirement (Moudgil et al., 1986). This observation is consistent with the recently reported results of Becker et al. (1986) which suggest that in vivo protein–DNA interactions in the glucocorticoid response element require the presence of the hormone. Willmann and Beato (1986), however, have reported that steroid-free glucocorticoid receptor can bind specifically to mouse mammary tumor virus (MMTV) DNA. The results presented in this study suggest, but do not prove, that calf uterine PR can be transformed in the absence of the steroid. It is, however, clear that the extent of transformation would be greater with the liganded receptor.

The nature of the ligand appears to play a significant role in the transformation of PR. Although agonist-occupied PR could be transformed by different agents which are known to induce transformation in other receptor systems, its ability to undergo transformation was noticeably reduced when it was occupied by the known antiprogesterone RU486. Therefore, either the binding of RU486 with PR must result in protection of the group(s) or region(s) involved in the 8S to 4S transformation or the association of RU486 with PR is such that treatment with transforming agents causes dissociation of the steroid, making it difficult to identify the 4S form. This is supported by the data that show that the transformed receptor exhibits reduced or no binding to RU486. These observations also suggest that a change in the conformation of the hormone binding subunits may, therefore, result following transformation. This is reflected in the prolonged half-life of the steroid–ligand complex (Wolfson et al., 1980; Yang et al., 1982) as well as by the acquisition of affinity for nuclei and polyanions (Moudgil et al., 1981).

The DNA binding and sedimentation analysis of PR reported in this paper reveal different information. Although RU486-bound PR resists transformation to the 4S form, its DNA binding ability remains intact, at least to an appreciable degree. This means that either the two procedures measure different aspects of receptor transformation or incubation with DNA–cellulose may stabilize the heat-treated [^{3}H]RU486–receptor complex and thus prevent it from dissociation. The latter could contribute to the loss in the number of 8S [^{3}H]-RU486–receptor complexes during the ultracentrifugation analysis. This suggestion is consistent with the observations of Theofan and Notides (1984), who reported that the steroid binding site of calf uterine progesterone receptor is more readily inactivated by heat than is the DNA binding site. It was further suggested that nucleic acid binding induces a conformational change, which consequently restores the receptor's progesterone binding site to functional activity.

Over the past several years, different mechanisms have been proposed to explain the process of receptor transformation (Milgrom et al., 1973; Milgrom, 1981; Baulieu et al., 1983; Moudgil et al., 1985; Notides et al., 1985). Although transformation may involve proteolysis of a larger nontransformed form of GR (Vedeckis, 1983), chick oviduct PR transformation appears to be a direct consequence of dissociation of the oligomeric native molecule without involvement of enzyme-catalyzed steps (Baulieu et al., 1983). This assumption is further supported by the observation that transformation can be induced in purified chick oviduct PR (Puri & Toft, 1984) and that the molecular weight of the native receptor does not change upon transformation (Baulieu et al., 1983; Renoir et al., 1986). Once the oligomeric structure is disrupted, the agonist–receptor complexes may become active at the DNA level and bind to regulatory elements (E. E. Baulieu, personal communication; Von der Ahe et al., 1985, 1986).

The data reported in this paper favor, but do not prove, the hypothesis that PR transformation involves disruption of the oligomeric structure of PR (Renoir et al., 1984) involving separation of steroid binding subunits. RU486 binding to PR either causes a change in the conformation of the receptor that may disallow separation of subunits or may mask the region(s) of the receptor protein which is (are) sensitive to the transformation-inducing agents. Alternatively, RU486-bound receptor may undergo transformation which accelerates the dissociation of the steroid under the experimental conditions employed in this study. The latter view is supported by the demonstration of an adequate level of DNA–cellulose binding by the RU486-associated PR.

Finally, RU486 appears to be a ligand with potentially very diverse effects. Whereas transformation of [^{3}H]RU486-bound GR from thymocytes appeared to be defective (Philibert, 1984), comparable transformation and DNA binding have been reported with rat liver GR bound to agonist [^{3}H]triamcinolone acetonide or the antagonist [^{3}H]RU486 (Agarwal et al., 1985). Bourgeois et al. (1984) have reported that binding of RU486 to mouse GR results in decreased affinity for DNA in general and a reduced specific recognition of a site in the promoter region of MMTV proviral DNA. It was further suggested that the binding of RU486 to GR mimics pharmacologically the properties of a class of receptor variants (nt^-) which are nonfunctional and have reduced nuclear transfer and altered DNA binding capacity.

The clinical significance of antiprogesterone actions of RU486 is well established (Philibert, 1984; Baulieu, 1985), and a vast literature has also accumulated on the basic characterization of RU486-bound progesterone and glucocorticoid receptors (Philibert, 1984). The exact mechanism of action of RU486 has remained obscure. The data presented in this paper should aid in the thrust of attempts focused on identifying the underlying mechanism(s) of the antiproges-

tational actions of this important and newly synthesized steroid.

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Registry No. ATP, 56-65-5; KCl, 7447-40-7; progesterone, 57-83-0.

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