Ribonucleic Acid Is a Component of the Oligomeric, Transformed Mouse AtT-20 Cell Glucocorticoid Receptor†

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ABSTRACT: The glucocorticoid receptor from mouse AtT-20 pituitary tumor cells exists in three forms. The largest form is an untransformed (non-DNA-binding), oligomeric species (9.1 S, 8.3 nm, M_r 319000). Two transformed (DNA-binding) forms can be generated. One is an oligomeric protein (5.2 S, 6–8.3 nm, M_r 132000–182000), while the other is the monomeric, hormone-binding subunit (3.8 S, 6 nm, M_r 96000). The composition of the oligomeric, transformed receptor and its relationship to the monomeric protein were examined. The 3.8S monomer can be isolated from DEAE-cellulose (0.12 M step elution) in a form that continues to sediment at about 3.8 S on molybdate-containing sucrose gradients and at about 4.2 S on molybdate-free gradients. Addition of a non-hormone-binding component isolated from the same DEAE-cellulose column (0.5 M KCl step) can apparently interact with the 3.8–4.2 S monomer, increasing its sedimentation coefficient to 5.2 S (on molybdate-containing gradients) or 6.6 S (on low-salt, molybdate-free gradients). This factor is a macromolecule (nondialyzable) and is heat-stable (100 °C, 20 min). A dose-dependent shift to the higher sedimentation coefficient is observed when increasing quantities of the 0.5 M step material are added to the receptor monomer. This activity is abolished when the 0.5 M step material is treated with ribonuclease A. Further, when RNA is purified from the 0.5 M step by phenol/chloroform extraction, its ability to increase the S value of the monomer is retained. Ribonuclease treatment of the untransformed, 9.1S, oligomeric complex does not cause a significant decrease in sedimentation rate, while the same treatment of the 5.2S, oligomeric, transformed receptor (obtained after Sephadex G-25 transformation) causes a decrease in sedimentation rate to about 3.8 S. The addition of bovine liver mRNA and rRNA does not cause a shift in sedimentation rate of the receptor monomer to a discrete, higher sedimenting receptor form. However, the addition of total rabbit liver tRNA or three distinct tRNA species causes a shift in sedimentation to a similar, but not identical, form as that with the 0.5 M step material. We propose that the 5.2S, oligomeric transformed glucocorticoid receptor is composed of one monomeric hormone-binding, protein subunit (M_r 96000) and a low molecular weight RNA (M_r 36000). This interaction may be important for the role of the receptor in regulating gene expression.

After binding their ligands, steroid hormone receptor proteins are converted from a nonnuclear-binding and non-DNA-binding form to species that bind to genomic constituents. This process has been designated receptor transformation. It now seems likely that subunit dissociation is the molecular mechanism of steroid receptor transformation [reviewed in Vedeckis (1985)]. Using the mouse AtT-20 pituitary tumor cell line system, we have found that glucocorticoid receptor (GC-R)[1] transformation results in the dissociation of a 9.1S, oligomeric protein into two different transformed species with sedimentation coefficients of 5.2 and 3.8 S (Vedeckis, 1983b; Eastman-Reks et al., 1984; Reker et al., 1985a). When a wide variety of in vitro transformation protocols are used, including alkaline phosphatase treatment, the 5.2S oligomeric species is generated (Reker et al., 1985a,b). Generation of the 3.8S GC-R monomer in unfractionated cytosol requires the continued presence of high salt (0.3 M KCl) concentrations (Reker et al., 1985a), while the *partially purified* monomer (obtained after DEAE-cellulose chromatography) sediments at 3.8–4.2 S in low-salt gradients (Reker et al., 1985a; see below).

The exact molecular compositions of the 9.1S oligomeric, untransformed and 5.2S oligomeric, transformed GC-R species have been difficult to ascertain. Indeed, the molecular weight of the 5.2S transformed receptor cannot yet be unequivocally determined. For example, a molecular weight of about 182000 is obtained from the Stokes radius of samples run in parallel (8.3 nm) (Vedeckis, 1983b), while 132000 is obtained from the Stokes radius of samples subjected to sequential analysis (6 nm) (LaPointe & Vedeckis, 1984; Vedeckis, 1985; Reker et al., 1985a). The former molecular weight determination is consistent with the 5.2S receptor being a homodimer of 3.8S subunits, while the latter suggests an oligomer containing heterogeneous subunits. Both of these alternatives have been suggested for the structure of the mouse AtT-20 cell GC-R (Vedeckis, 1983b). The symmetry involved in a homotetramer–homodimer–monomer model of receptor structure is attractive, and this model has been espoused by a number of laboratories (Raaka & Samuels, 1983; Norris & Kohler, 1983; Vedeckis, 1983b; Holbrook et al., 1983; Sherman et al., 1983). However, others have suggested that non-hormone-binding proteins [receptor binding factors (RBFs) or "phantom" subunits] may be components of oligomeric forms

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[1] Abbreviations: GC-R, glucocorticoid receptor; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; Dex, dexamethasone (9α-fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione); Dex-M, dexamethasone 21-mesylate; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); DEPC, diethyl pyrocarbonate; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride.

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(Murayama et al., 1980a,b; Colvard & Wilson, 1981; Barnett et al., 1983; Radanyi et al., 1984; Joab et al., 1984). Some studies have also implicated a role for RNA in receptor structure [reviewed in Vedeckis (1985)]. Presented below are a detailed series of experiments that indicate that ribonucleic acid may be an integral component of the 5.2S oligomeric, transformed mouse GC-R.

Materials and Methods

Chemicals. [1,2,4,6,7-^{3}H]Dexamethasone (Dex), 78 Ci/mmol, and [6,7-^{3}H]triamcinolone acetonide (TA), 20 Ci/mmol, were obtained from Amersham. [6,7-^{3}H]Dexamethasone 21-mesylate (Dex-M), 48.9 Ci/mmol, was purchased from New England Nuclear. [2-^{14}C]Uridine (sp act. 51.2 mCi/mmol) was purchased from ICN Radiochemicals. Rabbit liver transfer RNA (type XII), bovine liver ribosomal RNA, trypsin, chymotrypsin, TPCK-treated trypsin, TLCK-treated α-chymotrypsin, bovine pancreatic ribonuclease A (RNase A) (type XII-A), sodium molybdate, diethyl pyrocarbonate (DEPC), soybean trypsin inhibitor (SBTI), and 1-thioglycerol were from Sigma. RNasin (human placental ribonuclease inhibitor) was obtained from Promega Biotec. Purified bovine liver total RNA and mRNA were generous gifts from Dr. Diana K. Sheiness (Department of Biochemistry, Louisiana State University Medical Center, New Orleans, LA). Tris and sucrose were "ultrapure" grade from Schwarz/Mann. All other chemicals were reagent-grade obtained from J. T. Baker.

Cell Culture. AtT-20 cells were grown and harvested as described previously (Vedeckis, 1981). To label the RNA of AtT-20 cells with [^{14}C]uridine, 100 mL of the cell suspension was transferred to a sterile 250-mL spinner flask and 50 μCi of [^{14}C]uridine was added for 8 h prior to preparing the cytosol.

Preparation of Cytosol and Labeling of Receptor. Preparation of cytosol from these cells with TETg buffer (20 mM Tris-HCl, pH 7.4 at 25 °C, 1 mM Na_2EDTA, 12 mM 1-thioglycerol) has been described in detail previously (Vedeckis, 1983b). To label the GC-R, cytosol was incubated with [^{3}H]Dex (3×10^{-8} M final concentration) overnight at 0–4 °C. The percentage of bound hormone was determined by a dextran–charcoal assay (Reker et al., 1985a) before each experiment, and it was always found to be 30–40% of the total added hormone.

Rapid separation of the bound hormone from the free steroid in cytosol was obtained with minimal dilution by a Sephadex LH-20 slurry adsorption procedure (Reker et al., 1985a). In some experiments, the method of transformation (e.g., Sephadex G-25 filtration) resulted in removal of the free hormone.

Preparation of 0.12 M and 0.5 M Steps. The preparation of the transformed receptor and non-hormone-binding macromolecule(s) was performed at 0–4 °C by using Sephadex G-25 gel filtration followed by DEAE-cellulose ion-exchange chromatography. Both of these procedures have been described previously (Vedeckis, 1983a,b). Typically, 6 mL of [^{3}H]Dex-labeled cytosol was applied to a 40-mL (packed volume) G-25 column, and 1-mL fractions were collected with TETg buffer to eluate the column. Receptor-containing fractions, monitored by radioactivity excluded from the column, were pooled. After 4 h of incubation at 0 °C, the pooled fractions were applied to a 6-mL DEAE-cellulose column equilibrated in TETg buffer. The column was washed with TETg buffer until 25 mL of drop-through were collected. The transformed receptor [which elutes from DEAE-cellulose columns at about 0.08 M KCl (Vedeckis, 1983b)] was then step-eluted with 25 mL of 0.12 M KCl in TETg buffer; the small portion of untransformed receptor and other non-hormone-binding macromolecules were subsequently eluted from the same column with 25 mL of 0.5 M KCl in TETg buffer. Again, 1-mL fractions were collected, and 20 μL of each fraction was counted for radioactivity. Receptor-containing fractions from each step elution were pooled and desalted by Sephadex G-25 chromatography. These pools are referred to as the 0.12 M step (transformed GC-R) and 0.5 M step (non-hormone-binding molecules). For practical reasons, many preparations of the 0.12 M and 0.5 M steps were stored at −70 °C for periods of 1 day to 2 months. This procedure caused no detectable alteration in the structure of the transformed receptor or non-hormone-binding macromolecule(s) nor any significant loss of the hormone-binding activity from the receptor protein.

Purification of Receptor Covalently Labeled with [^{3}H]-Dexamethasone 21-Mesylate. Cytosol was prepared as usual from 4 L of cell suspension, except that homogenization was performed in 3 volumes of TE buffer (TETg buffer minus the 1-thioglycerol). The GC-R was labeled with 4×10^{-7} M [^{3}H]Dex-M for 2 h at 0–4 °C, followed by inactivation of the unreacted Dex-M with 12 mM 1-thioglycerol, since free sulfhydryls react with Dex-M (Simons et al., 1983). [^{3}H]-Triamcinolone acetonide (TA, 5×10^{-8} M) was then added to label GC-R molecules that had not been covalently labeled with Dex-M.

The monomeric GC-R was purified by a modification of the method of Wrange et al. (1979). All columns were equilibrated with TETg buffer containing 1 mM PMSF (phenylmethanesulfonyl fluoride). Cytosol (13.5 mL) was applied to a 30-mL phosphocellulose (Whatman P-11) column connected in series to a 25-mL DNA–cellulose (P-L Biochemicals) column. The drop-through fractions containing radioactivity were collected, pooled, and made 0.3 M in KCl. Receptor transformation was allowed to proceed for 3 h at 0–4 °C. The sample was desalted by chromatography over a Sephadex G-25 column (250-mL packed volume). The pooled void volume fractions were made 5×10^{-9} M in [^{3}H]TA to preserve hormone binding activity. The sample was applied to a 35-mL DEAE–cellulose (DE-52) column and eluted with a linear salt gradient (0–0.45 M KCl). The transformed GC-R (which eluted at 0.085 M KCl) fractions were pooled, diluted to 0.05 M KCl with TETg buffer, and applied to a 10-mL DNA–cellulose column. The column was washed with 10 volumes of TETg–0.05 M KCl and 10 volumes of TETg–0.1 M KCl, followed by elution of the GC-R with TETg–0.225 M KCl. These buffers also contained 100 μg/mL insulin.

The purified GC-R was precipitated by the addition of 10 μL/mL 2% deoxycholate and 0.1 volume of 100% trichloroacetic acid. After a 30-min incubation at 0–4 °C, precipitated protein was pelleted by centrifugation at $27000g_{max}$. The pellet was neutralized with 10 μL of 1 N NaOH and resuspended in 0.5 mL of TETg containing 0.1 M KCl and 20% glycerol. This sample was then stored at −20 °C.

RNA Preparation. RNA was extracted either from the 0.5 M step or from AtT-20 cell cytosol according to the method described by Maniatis et al. (1982), except for the addition of two more ethanol precipitations after the last treatment with phenol/chloroform. For quantitating the amount of RNA, we used spectrophotometric readings at 260 and 280 nm, assuming that 1 A_{260} unit corresponds approximately to 40 μg/mL RNA. An A_{260}/A_{280} of about 2.0 was routinely obtained, indicating that the RNA was pure. RNA was stored in 70% ethanol at −20 °C. Before each experiment, an aliquot of well-vortexed RNA suspension was transferred to an Eppendorf tube and centrifuged for 15 min. The RNA pellet

was washed twice with 75% ethanol and then dried by placing the sample in an evacuated desiccator for 20 min. RNA was dissolved in an appropriate volume of TETg buffer just prior to incubation with the GC-R. For all experiments utilizing RNA, sterile, essentially RNase-free glassware was used. All solutions used for the isolation of RNA were also sterile.

Sucrose Gradient Ultracentrifugation. Vertical-tube rotor sucrose gradients (5.2 mL, 5–20% w/v) were prepared in TETg buffer with or without 20 mM Na_2MoO_4 as described in Eastman-Reks et al. (1984). Sedimentation properties of the GC-R complexes were analyzed as described previously (Eastman-Reks et al., 1984; Reker et al., 1985a) with [^{14}C]methylated chymotrypsinogen A (2.6 S), ovalbumin (3.5 S), aldolase (7.9 S), and β-amylase (9.4 S) standards run in parallel tubes. Sucrose gradient profiles of the receptor complexes shown in the figures have been taken from one of three or more replicate experiments.

Polyacrylamide Gel Electrophoresis of Covalently Affinity-Labeled Receptor and Protein Standards. Samples were electrophoresed on SDS–polyacrylamide gels (Laemmli, 1970), with a 2.7% stacking gel and 7.9% running gel in a water-cooled Protean slab gel apparatus (Bio-Rad) at 40 V for 18 h. Parallel lanes contained high molecular weight protein standards (Bio-Rad). For protein fixation, gels were treated with 10% acetic acid for 30 min. The protein standard lane was sliced from the rest of the gel, fixed in 50% methanol–10% acetic acid and then stained with a Bio-Rad silver strain kit. The remainder of the gel was soaked in Fluoro-Hance (Research Products International), dried, and subjected to fluorography with Kodak X-Omat RP film and a single Cronex Lightning Plus intensifying screen (Dupont) at −70 °C for 10 days.

Liquid Scintillation Counting. Radioactivity was measured in a Beckman LS 7500 liquid scintillation spectrometer using Beckman Ready-Solv EP. The counting efficiency was approximately 35%.

Results

Alterations in the Sedimentation of the Monomeric Receptor Caused by a Non-Hormone-Binding Macromolecule. Previous studies have shown that the untransformed GC-R from AtT-20 cells is an oligomer with a sedimentation coefficient of 9.1 S (Eastman-Reks et al., 1984; Reker et al., 1985a). The untransformed GC-R can be converted to two transformed (DNA-binding) species, a 5.2S oligomeric receptor and a 3.8S monomeric protein. It has been porposed that the 5.2S oligomeric transformed receptor may be a dimeric complex of 3.8S subunits (Vedeckis, 1983b). Support for this possibility comes from the observation that the 5.2S form can be reconstituted from the 3.8S monomers isolated from high-salt sucrose gradients by removing the salt and recentrifugation on low-salt sucrose gradients (Reker et al., 1985a). An alternative possibility is that the 5.2S GC-R contains a 3.8S monomeric GC-R subunit plus some other non-hormone-binding component(s) (Vedeckis, 1985; Reker et al., 1985a). This is not in contradiction to the previously mentioned results, since a nonreceptor macromolecule could cosediment with the 3.8S monomer on high-salt sucrose gradients and reassociate with it upon salt removal. We wished to study the interconversion of the 3.8S monomer and 5.2S oligomer further to distinguish between these two possibilities.

Untransformed, [^{3}H]dexamethasone-labeled GC-R complexes were chromatographed over a Sephadex G-25 column (followed by a 4-h incubation at 0–4 °C). The GC-R-containing void-volume fractions were pooled and applied to a DEAE-cellulose column (as described in detail under Materials and Methods). The transformed receptor was then step-eluted with 0.12 M KCl (0.12 M step). The small amount of untransformed GC-R (which elutes from DEAE-cellulose at 0.2 M KCl), as well as other DEAE-cellulose-bound molecules, was subsequently eluted with 0.5 M KCl (0.5 M step). Both steps were then desalted. When the transformed receptor thus obtained (0.12 M step) was centrifuged on low-salt, molybdate-free sucrose gradients, it sedimented as a homogeneous peak at 4.2 S (Figure 1A). The 0.5 M step showed a very low radioactive content on sucrose gradients (Figure 1A). The amount of bound hormone in the 0.5 M step (i.e., the percentage of the receptor remaining untransformed) varied from experiment to experiment, depending on the efficiency of the G-25 transformation. However, very little receptor was detected after desalting the 0.5 M step, never exceeding 20% of the total amount of receptor-bound hormone when the 0.12 M and 0.5 M steps were mixed. When the 0.12 M and 0.5 M steps were mixed together and incubated for 15 min prior to loading onto low-salt, molybdate-free sucrose gradients, an

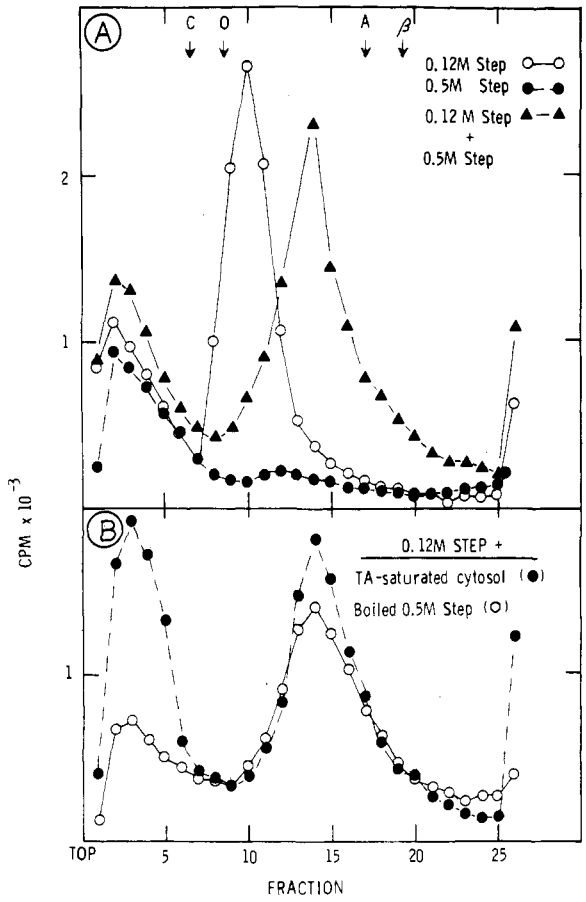

FIGURE 1: Interaction of a non-hormone-binding macromolecule(s) with the monomeric glucocorticoid receptor. AtT-20 cell cytosol labeled with [^{3}H]Dex was used to prepare monomeric, transformed receptor (0.12 M step) and non-hormone-binding macromolecules, (0.5 M step) from a DEAE-cellulose column, as described under Materials and Methods. In Panel A, 100 μL of 0.12 M step (○) and 0.5 M step (●) was diluted with an equal volume of TETg buffer or mixed together (▲). In Panel B, 100 μL of the 0.12 M step was mixed with 100 μL of the cytosol saturated with a 500-fold excess of radioinert triamcinolone acetonide (TA) (●) or with 100 μL of the 0.5 M step, which was boiled for 20 min (○). After a 15-min incubation at 0 °C, these samples (in both panels A and B) were analyzed on 5–20% low-salt (TETg = 20 mM Tris-HCl, pH 7.4 at 25 °C, 1 mM EDTA, 12 mM 1-thioglycerol), molybdate-free sucrose gradients at 2 °C in a vertical tube rotor. Standard proteins run on parallel gradients were chymotrypsinogen A (C, 2.6 S), ovalbumin (O, 3.5 S), aldolase (A, 7.9 S), and β-amylase (β, 9.4 S). The total time of the run was 79 min with a final $\omega^2 t$ of 3.04×10^{11} rad^2/s.

increase in the sedimentation value to 6.6 S was observed (Figure 1A). This suggested the existence of a factor(s) in the 0.5 M step that was (were) capable of interacting with the monomeric, transformed (4.2S) receptor. This association could be abolished by increasing the ionic strength of the medium. For example, if the 0.12 M step was mixed with the 0.5 M step and centrifuged on high-salt (0.3 M KCl) sucrose gradients, no increase in the sedimentation rate of the monomeric receptor was noted (data not shown).

The material in the 0.5 M step that caused an increased sedimentation rate when mixed with the GC-R monomer (0.12 M step) was further analyzed. It was found that the component(s) present in the 0.5 M step cannot bind radioactive Dex, even after a 12-h incubation. In addition, this component is nondialyzable (molecular weight cut-off = 10000–14000) and is heat-stable. When the 0.5 M step was boiled for 20 min, it was still able to effect the shift in GC-R sedimentation to 6.6 S (Figure 1B). The 0.5 M step is stable to freezing at −70 °C and could be freeze–thawed several times without destroying its ability to associate with the monomeric receptor. Also, after lyophilization the 0.5 M step material can still associate with the monomeric transformed receptor to yield an identical sucrose gradient profile as that obtained with fresh material.

As shown in Figure 1B, crude AtT-20 cell cytosol contains the component(s) that increase(s) the S value of the transformed receptor (4.2 S). That is, when the 0.12 M step is incubated with cytosol saturated with excess radioinert triamcinolone acetonide, the GC-R sediments as the same 6.6S species as when it is incubated with the 0.5 M step. Thus, the cytosolic component(s) is not noticeably altered by DEAE-cellulose chromatography.

To determine the kinetics of the formation of the 6.6S species, we incubated the 0.12 M and the 0.5 M steps for different time intervals (5–60 min). No difference could be seen between the 5- and 60-min incubation times (data not shown). This suggests that the association between the monomeric GC-R and the macromolecule(s) in the 0.5 M step is rapid. Thus, the macromolecule(s) may be present in high concentration and/or have a high affinity for binding to the transformed receptor. For all further experiments, we routinely used a 15-min incubation of the two DEAE-cellulose step elutions prior to analysis. The association is not affected by the presence of Na_2MoO_4, since the same increase in sedimentation rate can be obtained when the 0.5 M step is added to the 0.12 M step in the presence of 20 mM Na_2MoO_4 (data not shown).

A somewhat different picture is obtained if the same experiments described above are repeated on *molybdate-containing*, low-salt sucrose gradients (data not shown). Under these conditions, the monomeric GC-R (0.12 M step) sediments at 3.8 S, while a 5.2S GC-R species is obtained when the 0.12 M and 0.5 M steps are mixed. Thus, in molybdate-containing gradients, the sedimentation coefficients of the monomeric, transformed and reconstituted, oligomeric, transformed GC-R are the same as those obtained previously for the native forms on molybdate-containing gradients (Reker et al., 1985a).

Titration of the Monomeric Receptor with the 0.5 M Step Macromolecule(s). Having shown that the macromolecule(s) present in the 0.5 M step and in cytosol can, in a specific way, associate with the monomeric receptor, we wanted to determine if the appearance of the 6.6S species was dependent upon the concentration of the macromolecular component present in the 0.5 M step. Thus, a constant amount of monomeric receptor (0.12 M step) was titrated with increasing amounts of 0.5 M step. After the usual 15-min incubation, each sample was analyzed on low-salt sucrose gradients. The amounts of the transformed, monomeric receptor (4.2 S) and the 6.6S form were expressed as the percentage of total receptor-bound hormone (4.2 S + 6.6 S) obtained on the sucrose gradients. The amount of total receptor-bound hormone was not significantly different in all of these samples. An excellent correspondence was seen between the appearance of the 6.6S species and the disappearance of the 4.2S, transformed receptor, with increasing amounts of added 0.5 M step (data not shown). Virtually all of the 4.2S monomer could be converted into the 6.6S form when equal volumes of 0.12 M and 0.5 M steps were mixed. Most importantly, even at the high concentrations of added 0.5 M step, a discrete peak of 6.6S receptor was formed (data not shown). No intermediate peaks of hormone-binding activity were detected in any of these experiments. Since no higher sedimenting or intermediate aggregates were obtained, these results suggest that the association of the 4.2S monomer with the 0.5 M step material is of a specific nature.

Characterization of the 0.5 M Step Macromolecule(s). Partial characterization of the component present in the 0.5 M step revealed that it is a macromolecule that does not bind the hormone, it is heat-stable, and it has a net negative charge (judged by its strong adsorption to DEAE-cellulose). It has been reported that RNase treatment of steroid receptor complexes stimulates DNA–cellulose binding ability of receptors and also that the addition of RNA inhibits the DNA-binding activity of receptor (Feldman et al., 1981; Chong & Lippman, 1982; Tymoczko & Phillips, 1983; Tymoczko et al., 1984). On the basis of the aforementioned facts, we decided to investigate the possibility that the 6.6S form of the receptor could be generated by an association of the monomeric receptor with RNA present in the 0.5 M step (and in cytosol). We found that when the mixed 0.12 M and 0.5 M steps were treated with RNase A (90 units/mL, 60 min at 0 °C), no increase in S value to 6.6 S in low-salt gradients (Figure 2A) or to 5.2 S in molybdate-containing gradients (Figure 2B) occurred. The sedimentation position of the RNase-treated sample is the same as the position of the monomeric, transformed receptor to which no 0.5 M step was added (see Figure 1A). These results indicate that the 6.6S form of the receptor differs from the 4.2S receptor in that it contains an RNA component. In additional experiments, we examined the ability of different concentrations of RNase A (from 4.5 to 180 units/mL, 60 min at 0 °C) to alter the sedimentation profile of the 6.6S form. We found that under our experimental conditions RNase was fully active in destroying the ability of the 0.5 M step to increase the sedimentation rate of the monomer at concentrations greater than 45 units/mL (data not shown).

One possible explanation for the absence of any change in receptor sedimentation value in the RNase-treated sample is that the RNase A is contaminated with some proteolytic enzyme, in spite of the fact that the RNase A used for these experiments was preincubated in a boiling water bath for 15 min. That these effects were not due to proteolytic activity is further supported by the observation that neither leupeptin (3–5 mM) nor 20 mM molybdate had any inhibitory effect on the RNase-treated samples; a 4.2S GC-R form was still obtained (data not shown).

To test this further, we used a recently developed affinity label for glucocorticoid receptors, namely, dexamethasone 21-mesylate (Simons et al., 1983). The covalently labeled, purified GC-R was prepared as described under Materials and

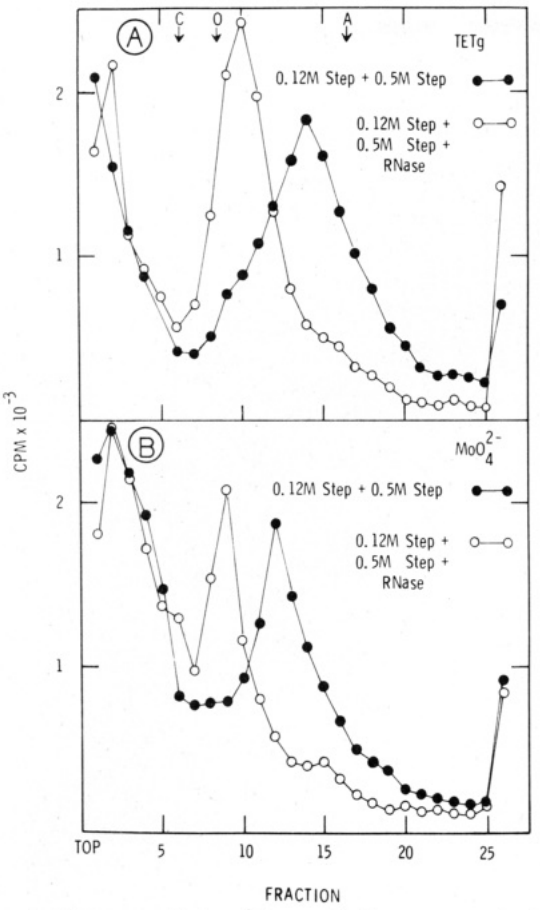

FIGURE 2: RNase sensitivity of the non-binding macromolecule(s). Five hundred microliters of the 0.12 M step was added to 500 μL of the 0.5 M step (●). After 15-min incubation at 0 °C, half of the mixture was treated with RNase A (90 units/mL) for 60 min at 0 °C (○). Two hundered microliters of each sample was then centrifuged on either low-salt (TETg), molybdate-free gradients (panel A) or low-salt (TETg) gradients containing 20 mM Na_2MoO_4 (panel B). Standard proteins and conditions are the same as indicated in the legend to Figure 1.

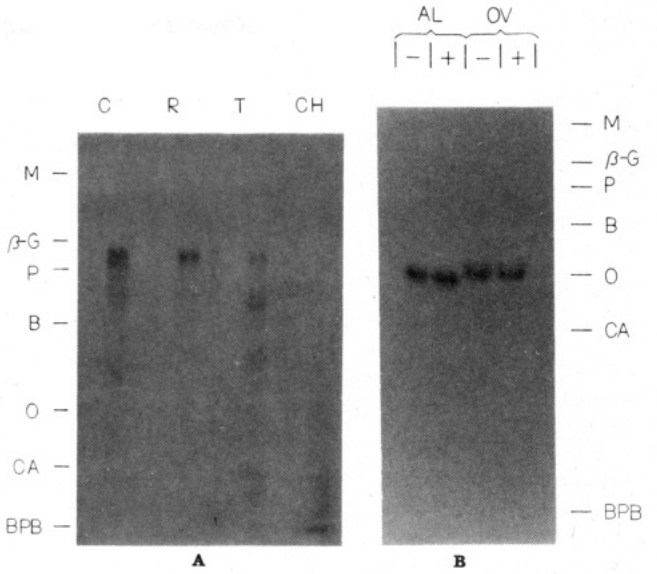

FIGURE 3: Lack of detectable glucocorticoid receptor and protein standard proteolysis after RNase A treatment. (A) [^{3}H]Dexamethasone 21-mesylate (Dex-M) affinity-labeled receptor was purified from mouse AtT-20 cells as described under Materials and Methods. Aliquots were incubated with buffer alone (C = control), with 90 units/mL RNase A (R), with 0.05 mg/mL TPCK-treated trypsin (T), or with 0.05 mg/mL TLCK-treated α-chymotrypsin (CH), all for 1 h at 0 °C. (B) [^{14}C]Methylated aldolase (AL) and ovalbumin (OV) (~10000 cpm each) were incubated for 1 h at 0 °C with buffer alone [(−) lanes] or with 90 units/mL RNase A [(+) lanes]. SDS–PAGE of all samples was performed as described under Materials and Methods. Standard proteins were run in parallel lanes and stained by silver staining. The standards used were myosin (M, M_r 200000), β-galactosidase (β-G, M_r 116250), phosphorylase B (P, M_r 97500), bovine serum albumin (B, M_r 66200), ovalbumin (O, M_r 45000), and carbonic anhydrase (CA, M_r 31000). BPB = bromphenol blue dye front.

Methods and was treated with pancreatic RNase A under standard reaction conditions (90 units/mL, 1 h, 0 °C). Control GC-R (without RNase) and RNase-treated GC-R, as well as trypsin- and chymotrypsin-treated GC-R samples, were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The fluorograph in Figure 3A shows that in the control sample only one protein, with a molecular weight of about 100000, is labeled. This corresponds well to the molecular weight of the 3.8S, hormone-binding monomeric GC-R. When [^{3}H]Dex-M labeled receptor was treated with RNase A and analyzed by SDS–PAGE, no substantial difference could be seen in the size of the 100K moiety or its intensity of labeling. That the purified, [^{3}H]Dex-M-labeled receptor is susceptible to proteolysis is also shown in Figure 3A; trypsin and chymotrypsin treatment caused extensive receptor fragmentation. Finally, RNase A treatment of [^{14}C]methylated aldolase and ovalbumin did not cause a decrease in the size of these standard proteins when analyzed on SDS–PAGE (Figure 3B). Thus, it seems unlikely that the RNase preparation contains any significant amount of protease activity. These studies strongly suggest that the added ribonuclease activity (and not a contaminating protease) destroys the ability of the 0.5 M step macromolecules to increase the sedimentation rate of the GC-R monomer.

Further studies were performed to confirm that the 0.5 M step material that caused increased GC-R sedimentation was not proteinaceous. Initial studies showed that treatment of the 0.5 M step material with trypsin seemed to destroy the ability to shift the GC-R from 4.2 to 6.6 S (Table I, protocol 1). This might indicate that the 0.5 M step material is a protein. However, since the tissue source of both trypsin and RNase A was bovine pancreas, an alternative possibility was that the added trypsin was contaminated with ribonuclease. Therefore, the trypsin enzyme preparation was pretreated with two ribonuclease inhibitors, RNasin (Table I, protocol 2) and DEPC (Table I, protocol 3), prior to the addition of the 0.5 M step material. Under these conditions, the trypsin-treated 0.5 M step material was fully active in shifting the 4.2S GC-R monomer to 6.6 S. Suitable controls showed that RNasin alone does not alter the sedimentation position of the 4.2S GC-R and that neither RNasin nor DEPC has any trypsin-inhibiting activity, since trypsin was capable of proteolyzing the 4.2S GC-R monomer to the 2.6S mero-receptor (Vedeckis, 1983a) in their presence (Table I). All of the studies mentioned above support the notion that the macromolecular factor in the 0.5 M step that interacts with the 4.2S GC-R monomer to shift its sedimentation rate to 6.6 S is, indeed, ribonucleic acid.

RNase Sensitivity of the Native Receptor Species. We have previously shown (Reker et al., 1985a,b) that a wide variety of in vitro and in vivo transformation protocols yield the 5.2S GC-R form when analyzed on MoO_4^{2-}-containing gradients or the 6.6S form on low-salt, molybdate-free sucrose gradients. We have also shown in Figures 1 and 2 that when the 0.12 M and 0.5 M steps are mixed together, sedimentation positions very similar to these are obtained. The clear implication, then, is that the intermediate, oligomeric, transformed receptor

Table I: Protease Resistance of the 0.5 M Step Material

	treatments[a]	receptor form
protocol 1	0.5 M step + trypsin[b] $\xrightarrow{60\text{ min}}$ + SBTI[c] $\xrightarrow{30\text{ min}}$ + 0.12 M step $\xrightarrow{15\text{ min}}$	4.2 S
protocol 2	trypsin + RNasin[d] $\xrightarrow{10\text{ min}}$ + 0.5 M step $\xrightarrow{60\text{ min}}$ + SBTI $\xrightarrow{30\text{ min}}$ + 0.12 M step $\xrightarrow{15\text{ min}}$	6.6 S
protocol 3	trypsin + DEPC[e] $\xrightarrow{10\text{ min}}$ + 0.5 M step $\xrightarrow{60\text{ min}}$ + SBTI $\xrightarrow{30\text{ min}}$ + 0.12 M step $\xrightarrow{15\text{ min}}$	6.6 S
controls	(a) 0.12 M step + RNasin $\xrightarrow{60\text{ min}}$	4.2 S
	(b) 0.12 M step + trypsin $\xrightarrow{60\text{ min}}$	2.6 S
	(c) 0.12 M step + trypsin + RNasin $\xrightarrow{60\text{ min}}$	2.6 S
	(d) 0.12 M step + trypsin + DEPC $\xrightarrow{60\text{ min}}$	2.6 S

[a] All incubations were performed at 0 °C. [b] The trypsin concentration used in these experiments was 50 μg/mL. [c] SBTI = soybean trypsin inhibitor (500 μg/mL). [d] RNasin = human placental ribonuclease inhibitor (2000 units/mL). [e] DEPC = diethyl pyrocarbonate (1%).

represents the monomeric, 3.8S form associated with an RNA molecule(s). To further test this observation, we examined the ability of RNase to convert the native (as opposed to reconstituted) 5.2S form into the 3.8S transformed receptor. The intermediate, oligomeric transformed receptor was obtained by chromatography of AtT-20 cell cytosol over Sephadex G-25 (followed by a 4-h incubation at 0–4 °C). The GC-R was then analyzed on MoO_4^{2-}-containing gradients (Figure 4A). As we have observed previously, the transformed receptor migrated as a sharp peak in the 5.2S region of the gradient. In addition, a sharp 9.1S, untransformed receptor peak was obtained. When the same sample was treated for 60 min at 0 °C with RNase A, the 5.2S peak was converted to a 3.8S form (Figure 4A), while the position of the 9.1S untransformed receptor remained unchanged after RNase treatment. Companion samples were also analyzed on low-salt (TETg) gradients (Figure 4B). The G-25-transformed receptor sedimented predominantly as a 6.6S species, with a small shoulder in the 9.1S region. Upon RNase treatment, two distinct peaks, 4.2 and 8.8 S, were obtained. The same pattern was obtained when either leupeptin or MoO_4^{2-} was included in the RNase incubation mixture (data not shown). Similar experiments performed by Tymoczko & Phillips (1983) showed that MoO_4^{2-} inhibited the decrease in receptor sedimentation upon RNase treatment. The reason for this discrepancy with our results is not yet clear. In any event, the above results suggest that the native, G-25-transformed receptor obtained from cytosol contains an RNA component that binds to the monomeric transformed receptor in hypotonic buffer (with or without 20 mM MoO_4^{2-}) and that it can be hydrolyzed by RNase treatment.

The results in Figure 4 seemed to indicate that the 9.1S oligomeric, untransformed GC-R was relatively unaffected by RNase A treatment. To further investigate this, we performed an analysis on cytosolic GC-R that had not been transformed. Analysis of the untransformed glucocorticoid receptor by sucrose gradient ultracentrifugation demonstrated a 9.1S oligomeric receptor plus a small portion of transformed receptor in the 5.2S region of the sucrose gradient (Figure 5). RNase treatment of this preparation had no effect on the sedimentation value of the untransformed receptor, while the 5.2S form shifted to a lower S value. The 9.1S peak of radioactivity was observed regardless of whether or not the MoO_4^{2-} was present during the RNase treatment (Figure 5). Although it has been reported that RNase can interact directly with the estrogen receptor, increasing the sedimentation coefficient from 9.7 to 10.4 S (Feldman et al., 1983), we did not notice any increase in the S value of the untransformed GC-R upon RNase treatment. Insensitivity of the 9.1S receptor to RNase suggests two possibilities. First, RNA may not be associated with the

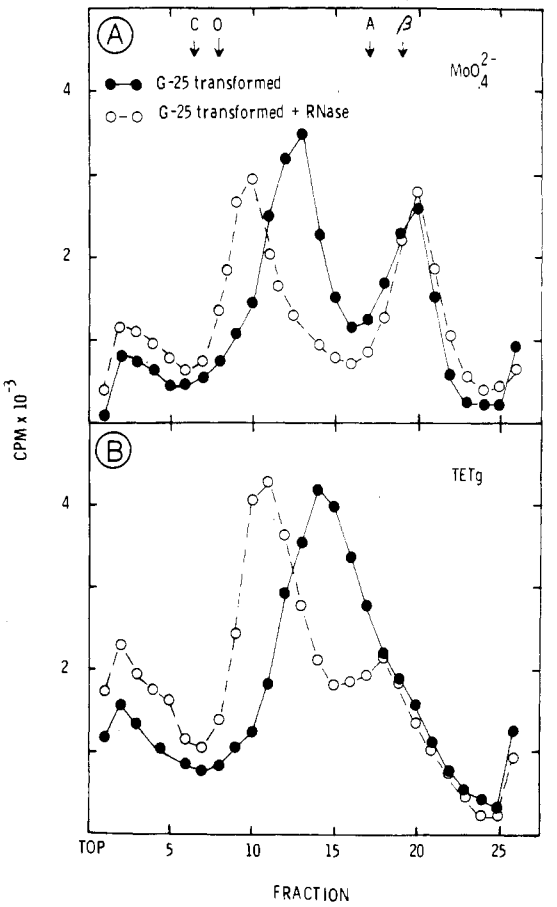

FIGURE 4: Conversion of the intermediate, oligomeric transformed receptor to the transformed monomer by RNase. [^{3}H]Dex-labeled cytosol was transformed by Sephadex G-25 chromatography, followed by a 4-h incubation at 0 °C (●). Transformed glucocorticoid receptor was then treated with RNase A (90 units/mL) for 60 min at 0 °C (○). Aliquots (200 μL) of untreated and RNase-treated transformed receptor were then centrifuged on MoO_4^{2-}-containing (panel A) or low-salt, molybdate-free (TETg, panel B) vertical-tube rotor sucrose gradients. Standard proteins used are the same as indicated in the legend to Figure 1.

untransformed receptor. Alternatively, it is conceivable that there may be endogenous RNA present that is protected by the receptor protein and is, therefore, inaccessible to RNase digestion. To analyze this possibility, we labeled cellular RNA with [^{14}C]uridine. We reasoned that if RNA were associated with the 9.1S, untransformed GC-R, a peak a ^{14}C-labeled RNA would cosediment with the receptor but be resistant to RNase treatment.

AtT-20 cells were incubated with [^{14}C]uridine to label the cellular RNA as described under Materials and Methods.

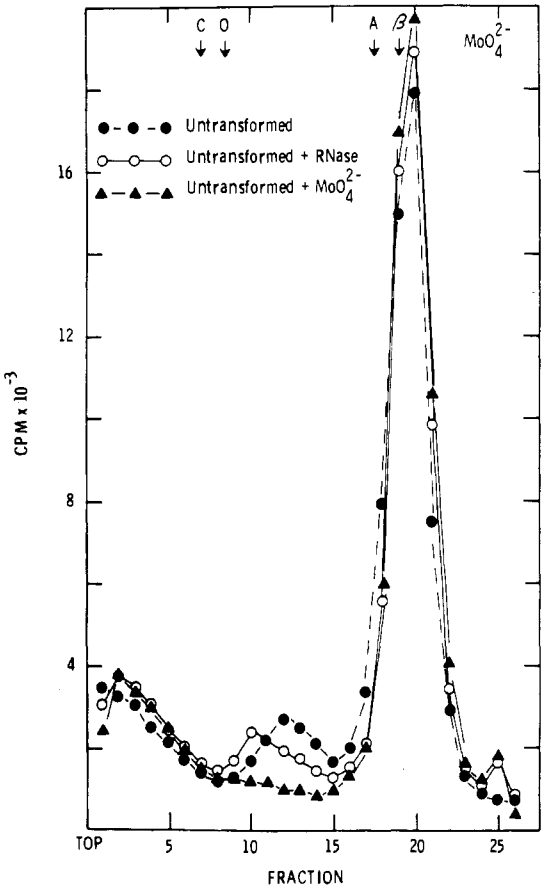

FIGURE 5: Insensitivity of the 9.1S, untransformed receptor to RNase A. [^{3}H]Dex-labeled cytosol (which contains untransformed glucocorticoid receptor) was treated with RNase A (90 units/mL) in the absence (○) or presence (▲) of 20 mM Na_2MoO_4 for 60 min at 0 °C. Another identical sample was kept on ice (●). Two hundred microliters of each sample were then analyzed on MoO_4^{2-}-containing sucrose gradients. The arrows indicate the sedimentation position of protein standards as described in the legend to Figure 1.

Cytosol was then prepared and incubated with [^{3}H]Dex as usual. This preparation was then divided in half. One portion was incubated (1 h, 0 °C) with 90 units/mL RNase A while the same volume of buffer was added to the other as a control. Sucrose gradient ultracentrifugation in low-salt, molybdate-containing buffer was used to analyze both samples. The untransformed receptor sedimented at 9.1 S, as expected. No *discrete* peak of ^{14}C-labeled RNA cosedimented with the 9.1S GC-R, although some ^{14}C-labeled RNA clearly did sediment to the 8–10S region in the gradient (Figure 6A). RNase treatment eliminated the ^{14}C-labeled RNA in this region, while the sedimentation characteristics of the 9.1S GC-R were unaltered (Figure 6B). This shows that the ^{14}C-labeled RNA that cosedimented with 9.1S GC-R was not protected from hydrolysis. These results (and those shown in Figure 5) suggest that RNA is not a part of the untransformed GC-R. However, it still is possible that the RNA is associated with the 9.1S untransformed GC-R. Perhaps it is not tightly associated with the untransformed receptor or is present in too small an amount to be detected by in vivo [^{14}C]uridine labeling as described here. Also, the turnover of the RNA associated with the oligomeric, untransformed GC-R may be too slow to allow its labeling during the 8-h incubation period with [^{14}C]uridine. More sensitive techniques are needed to give a definitive answer to this question. Indeed, results obtained from other laboratories suggest that RNA may be present in the untransformed GC-R complex (see Discussion).

Effects of Various RNA Species on the Sedimentation of the Monomeric Receptor Protein. We have attempted some preliminary characterization of the RNA that apparently associates with the monomeric GC-R. The main goal was to determine if some specificity exists for this association. When purified bovine liver rRNA or mRNA was added to the transformed monomer (0.12 M step), no discrete shift in sedimentation to the 6.6S species was observed (data not shown). On the other hand, a shift to an approximately 6S species was obtained with the addition of total RNA isolated from bovine liver (data not shown). This suggests that some RNA species present in total RNA (but not mRNA or rRNA) is capable of associating with the monomer to yield this 6S form.

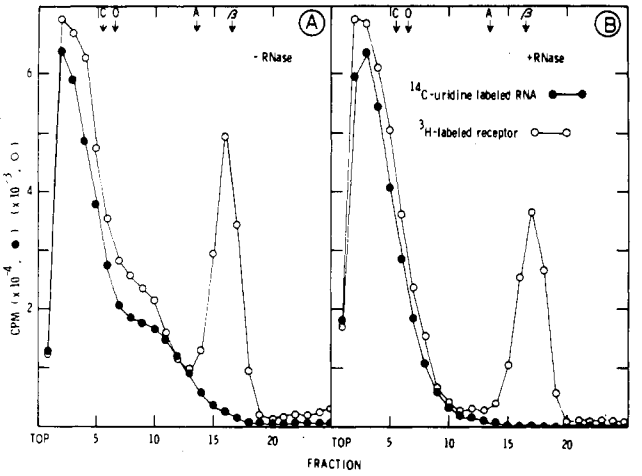

FIGURE 6: Sedimentation of RNA and the untransformed glucocorticoid receptor before and after RNase A treatment. AtT-20 cells were incubated with [^{14}C]uridine to label cellular RNA (●) as described under Materials and Methods. The cytosol was prepared and incubated with [^{3}H]Dex for 2 h at 0 °C (○). At the end of the incubation, cytosol was split into two aliquots. One aliquot was left untreated (panel A) while the other was incubated with RNase A (90 units/mL) for 60 min at 0 °C (panel B). Two hundred microliters of each sample were applied to low-salt (TETg) molybdate-free sucrose gradients (5–20%) and centrifuged under the same conditions as described in the legend to Figure 1.

We also wanted to test whether RNA purified from the 0.5 M step could associate with the transformed monomer to cause the shift in the S value. Therefore, RNA was extracted from the 0.5 M step and added to the monomer (0.12 M step) in the presence or absence of RNase. Samples were then analyzed by sucrose gradient ultracentrifugation (Figure 7A). Addition of this purified RNA caused a shift in the S value to about 6.3 S, and RNase treatment prevented this shift. In addition, tRNA from rabbit liver was added to the monomer in either the presence or absence of RNase. Samples were then subjected to sucrose gradient analysis. Results shown in Figure 7B indicate that the monomeric receptor is also capable of binding to tRNA. Association with tRNA increased the S value of the monomeric receptor to about 5.7 S in low-salt molybdate-free gradients, and this is prevented by RNase treatment. This is particularly interesting since we believe that the oligomeric, transformed form of the GC-R represents a complex of the monomeric transformed receptor with some small RNA (transfer RNA or some other small RNA) present in cytosol. In both panels A and B of Figure 7, some heavier aggregates can also be seen. We do not know whether this was due to impurities in the RNA purified from the 0.5 M step and in the tRNA mixture. A more discrete sedimentation profile was obtained when the 0.12 M step was incubated with specific tRNAs (valine, phenylalanine, and glutamic acid tRNAs), and the heavier aggregate forms of

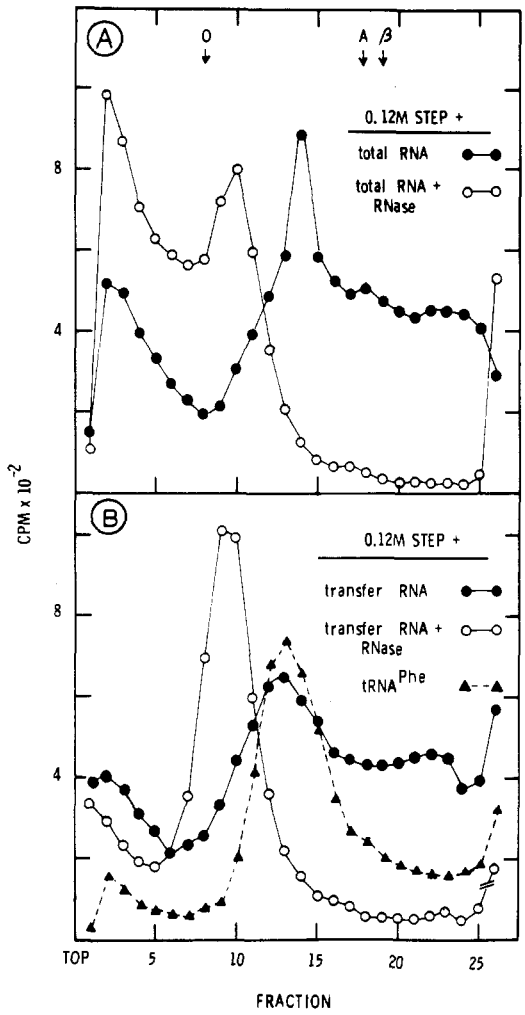

FIGURE 7: Effect of various RNA species on monomeric glucocorticoid receptor sedimentation rate. (Panel A) A total of 120 μg of total RNA purified from the 0.5 M step material obtained from AtT-20 cell cytosol was dissolved in 400 μL of 0.12 M step material. Half of this was left untreated (●) while the other half was incubated with RNase A (90 units/mL) for 1 h at 0 °C (○). (Panel B) A total of 120 μg of total rabbit liver tRNA was dissolved in 400 μL of 0.12 M step material. Half of the sample was left untreated (●) while the other half was treated with RNase A as described for panel A (○). Also, 60 μg of phenylalanine tRNA purified from *Escherichia coli* was dissolved in 200 μL of 0.12 M step material (▲). Two hundred microliters of each of the above samples was analyzed by low-salt (TETg) sucrose gradient ultracentrifugation. The remainder of the details are given under Materials and Methods and in the legend to Figure 1.

the GC-R did not appear (Figure 7B). Since it has been reported that steroids can bind to aminoacyl transfer RNA (Chin & Kidson, 1971), we wanted to ascertain whether tRNA–dexamethasone entities might be formed under our conditions. When tRNA was incubated with [^{3}H]Dex for 2 h, no dexamethasone binding could be detected on low-salt sucrose gradients (data not shown). Therefore, the presence of Dex–tRNA complexes can be excluded. The interaction between tRNA and the monomeric receptor is being further investigated in our laboratory.

The experiments described above show that *purified* RNA can interact with the GC-R monomer to cause an increase in sedimentation rate. Discrete peaks of GC-R can be obtained with the addition of bovine liver total RNA, tRNA (either total or individual types), and AtT-20 cell RNA purified from the 0.5 M step material. However, it is important to note that only RNA isolated from the 0.5 M step gave identical S value (5.2 S, 6.6 S) to those obtained when receptors are subjected to in vitro and in vivo transformation. The other heterologous RNAs added to the monomer caused a slightly lower, but consistent, shift in sedimentation coefficient. Thus, we believe that the 5.2S (6.6S) transformed GC-R contains a small RNA molecule that may be similar, but not identical, in size to transfer RNA.

Discussion

The possible role of RNA in steroid hormone receptor structure was first postulated over 10 years ago by Liao et al. (1973), for both the estrogen and androgen receptor. In spite of this, only a rather limited number of studies on the role of RNA in receptor structure have appeared in the ensuing years. However, RNA has been implicated in the structure of many steroid receptors, including those for glucocorticoids (Liao et al., 1980; Chong & Lippman, 1982; Hutchens et al., 1982; Rossini & Barbiroli, 1983; Tymoczko & Phillips, 1983; Tymoczko et al., 1984), estrogens (Liao et al., 1973, 1980; Liang & Liao, 1974; Chong & Lippman, 1982), androgens (Liao et al., 1973, 1980; Haase et al., 1983), progestins (Liao et al., 1980), and the sterol vitamin D (Franceschi & DeLuca, 1979; Franceschi et al., 1983; Franceschi, 1984). Most of these studies have involved treating crude cytosolic receptors with ribonuclease and observing a shift in receptor sedimentation rate or a change in DNA–cellulose binding activity. A more recent study shows that, after chemical cross-linking, the buoyant density of the GC-R is that of a ribonucleoprotein (Economidis & Rousseau, 1985). We have also found that the addition of purified cellular RNA to a partially purified, monomeric receptor causes a discrete shift in sedimentation position either from 3.8 to 5.2 S (molybdate-containing gradients) or from 4.2 to 6.6 S (low-salt, molybdate-free gradients). Most significantly, these higher sedimentation coefficients are identical with those obtained when the 9.1S, oligomeric, untransformed receptor is transformed by various experimental means. Thus, we may have succeeded in reconstituting the same oligomeric, transformed GC-R that is obtained with either in vitro or in vivo GC-R transformation (Reker et al., 1985a). Therefore, it is likely that a low molecular weight RNA is a component of the 5.2S (or 6.6S) oligomeric, transformed GC-R.

A determination of the authentic molecular weight of the 5.2S, oligomeric, transformed GC-R has been difficult, largely for technical reasons [discussed in Vedeckis (1985) and in Reker et al. (1985a)]. Although a dimeric structure comprised of identical 3.8S, hormone-binding, monomeric subunits is the most conceptually attractive hypothesis (Raaka & Samuels, 1983; Norris & Kohler, 1983; Vedeckis, 1983b; Holbrook et al., 1983; Sherman et al., 1983), this structure has not been unequivocally demonstrated. Our current results support an alternative model for the structure of this species, that is, a 3.8S, hormone-binding monomeric protein (M_r 96 000) plus an RNA molecule (M_r 36 000).

The preliminary experiments presented here do not allow us to draw any firm conclusions about the structure of the 9.1S, oligomeric, untransformed, GC-R species. However, when the untransformed rat liver GC-R receptor was purified in the presence of sodium molybdate, four bands of silver-staining material were observed on SDS–polyacrylamide gels (Grandics et al., 1984a). The molecular weights of these macromolecules were about 90K, 41K, 40K, and 24K. Subsequent studies suggested that the 41K and 40K proteins were proteolytic products of the 90K protein (Grandics et al., 1984b). When this experiment was repeated with animals injected with [^{32}P]orthophosphate, two radioactively labeled bands were obtained on SDS gels at 90K and 24K. The latter component

was not detected by Coomassie Blue staining of the gel (Grandics et al., 1984b). Similar results were obtained in a study using L cells (Housley & Pratt, 1984). In addition, RNase treatment of the molybdate-stabilized, purified, untransformed L cell GC-R did not affect the phosphorylated 92K band (GC-R monomer), while a ^{32}P-labeled 21K band was eliminated by this treatment. Preliminary studies with AtT-20 cells in our laboratory have given identical results (B. Kovačič-Milivojević and W. V. Vedeckis, unpublished results). These studies demonstrate that a low molecular weight RNA might copurify with the molybdate-stabilized, untransformed GC-R. This suggests, but does not prove, that a low molecular weight RNA may be a structural component of the oligomeric, untransformed receptor.

Further studies are required to determine if this low molecular weight RNA is of physiological relevance. One possibility is that the untransformed receptor is an oligomer containing only proteinaceous subunits, which dissociate into monomeric, hormone-binding subunits upon receptor transformation. Since this exposes the DNA binding site of the monomer, it is possible that the receptor then associates with a soluble RNA in the cytosolic extract. Thus, GC-R transformation, in this case, would follow the sequence 9.1 S → 3.8 S → 5.2 S. Indeed, this could also be the basis for the observed 4S → 5S estrogen receptor transformation [discussed in Vedeckis (1985)]. Alternatively, if the RNA is a component of the native 9.1S, untransformed species, the transformation reaction should procede 9.1 S → 5.2 S → 3.8 S. Kinetic studies to distinguish between these two models are currently in progress. In addition, we have not yet determined if the RNA-binding site is identical with the DNA binding site.

The discrete, symmetrical 5.2S (or 6.6S) peak observed upon addition of the cytoplasmic RNA to the 3.8S monomer argues against a totally nonspecific adsorption of the receptor to RNA. Therefore, it is quite possible that the oligomeric, transformed (and, perhaps, untransformed) receptor is interacting with a specific low molecular weight RNA. Although the addition of tRNA results in a shift of the monomeric receptor to a higher S value (5.7 S) on low-salt, molybdate-free gradients, it is clearly not the same as that obtained with the purified cytosolic RNA (6.6 S). This may indicate that the cytosolic RNA is "tRNA-like", perhaps having extensive secondary structure. Alternatively, perhaps the shift observed with tRNA is merely fortuitous and based upon the relatively homogeneous sedimentation nature of the tRNA preparations used.

Finally, what physiological role might receptor binding to RNA have? At the present time, any suggestions in this regard must necessarily be speculative. One possibility is that RNA (by interacting simultaneously with the DNA binding sites of receptor monomers) stabilizes the oligomeric, untransformed receptor complex. Another possibility is that binding of the receptor to RNA helps stabilize various RNA species. For example, although the primary effects of steroid receptor complexes are believed to occur at the level of mRNA transcription, steroid hormones also stimulate protein synthesis posttranscriptionally (Robins & Schimke, 1978; McKnight & Palmiter, 1979; Page & Parker, 1982). This could be due to the specific binding of the receptor to hormonally regulated mRNAs, resulting in mRNA stabilization. Similarly, it is possible that the RNA-binding activity is involved in autogenous regulation of gene transcription. For example, if steroid receptors bind specifically to hormonally induced mRNAs, this might reduce the amount of receptor that can interact with the hormonally regulated gene, thereby decreasing its transcription when mRNA levels are high. An even more exciting possibility is that the RNA is an integral component of the receptor and that it has homology to the control regions of hormonally regulated genes. Hybridization of the RNA to these gene sequences might destabilize the DNA double helix, thereby rendering transcription of these genes more efficient. All of the above-mentioned possibilities require that a specific RNA interacts with the receptor. Characterization of this RNA is presently being pursued in our laboratory to address this question.

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Fluorescent Analogues of *N,N'*-Dicyclohexylcarbodiimide as Structural Probes of the Bovine Mitochondrial Proton Channel†

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ABSTRACT: *N*-Cyclohexyl-*N'*-[4-(dimethylamino)-α-naphthyl]carbodiimide (NCD-4) and *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide (NCP) are two novel fluorescent analogues of the mitochondrial inhibitor dicyclohexylcarbodiimide (DCCD). Although nonfluorescent in aqueous media, both compounds form fluorescent conjugates with mitochondrial electron transport particles (ETP_H) or purified H^+-ATPase (F_1-F_0) vesicles. DCCD prevents the reaction of ETP_H with both NCD-4 and NCP. The fluorescent probes are effective inhibitors of ATPase activity and ATP-driven membrane potential, although their reaction rates are considerably slower than that of DCCD. The fluorescence of NCD-4- or NCP-treated H^+-ATPase is quenched by hydrophobic spin-label nitroxide derivatives of stearic acid (*x*-NS) in the order 16-NS > 12-NS > 7-NS ≃ 5-NS, whereas membrane-impermeant iodide ions have negligible effect. The quenching behavior of 16-NS (the most effective quencher) suggests that a small fraction of labels remain inaccessible to the quencher. It is concluded that the DCCD-binding sites are oriented toward the membrane lipids and are located in the lipid bilayer ca. 18 Å from the membrane surface.

The mitochondrial inhibitor dicyclohexylcarbodiimide (DCCD)[1] is a highly specific inhibitor of ATPase activity and ATP-driven proton translocation in both eukaryotic and prokaryotic H^+-ATPases (Catell et al., 1971; Linnet & Beechey, 1979). In all cases, the mechanism of inhibition involves the selective modification of a glutamic acid residue (aspartic in *Escherichia coli*) in the so-called proteolipid subunit of the H^+-ATPase complex (Sebald et al., 1980; Sebald & Wachter, 1980; Hoppe et al., 1980).

The proteolipid, or DCCD-binding protein, is a hydrophobic protein of molecular weight ca. 8000. It forms part of the membrane sector (F_0) of the H^+-ATPase (F_1-F_0) and is believed to function as the proton-conducting element or channel (Criddle et al., 1977; Moran et al., 1980; Celis, 1980). In mitochondrial and chloroplast F_0, there are probably six copies of the proteolipid (Sigrist-Nelson et al., 1978; Sebald et al., 1979) although the modification of only one subunit is sufficient for complete loss of ATPase activity (Sebald et al., 1976; Graf & Sebald, 1978), suggesting that the six monomers function in a concerted manner (Friedl et al., 1980).

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[1] Abbreviations: DCCD, dicyclohexylcarbodiimide; ATPase, adenosinetriphosphatase; NCD-4, *N*-cyclohexyl-*N'*-[4-(dimethylamino)-α-naphthyl]carbodiimide; NCP, *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide; ETP_H, mitochondrial electron transport particles; oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl)-2,4-pentadienylideneoxonol; *x*-NS, *x*-(4,4-dimethyl-*N*-oxyoxazolidin-3-yl)stearic acid (*x* = 5, 7, 12, 16); TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; NADH, reduced nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane.

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