Klappe, K., Wilschut, J., Nir, S., & Hoekstra, D. (1986) *Biochemistry* (preceding paper in this issue).
Klose, G., Arnold, K., Peinel, G., Binder, H, & Gawrisch, K. (1985) *Colloids Surf. 14*, 21–30.
MacDonald, R. I. (1985) *Biochemistry 24*, 4058–4066.
MacDonald, R. I., & MacDonald, R. C. (1983) *Biochim. Biophys. Acta 735*, 243–251.
Nir, S., Bentz, J., & Portis, A. (1980) *Adv. Chem. Ser. No. 188*, 75–106.
Nir, S., Wilschut, J., & Bentz, J. (1982) *Biochim. Biophys. Acta 688*, 275–278.
Nir, S., Bentz, J., Wilschut, J., & Düzgünes, N. (1983) *Prog. Surf. Sci. 13*, 1–124.
Nir, S., Stegmann, T., & Wilschut, J. (1986a) *Biochemistry 25*, 257–266.
Nir, S., Klappe, K., & Hoekstra, D. (1986b) *Biochemistry 25*, 2155–2161.
Parsegian, V. A., & Rand, R. P. (1983) *Ann. N.Y. Acad. Sci. 416*, 1–11.
Rand, R. P. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 277–314.
Richardson, C. D., & Choppin, P. W. (1983) *Virology 131*, 518–532.
Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1985) *Biochemistry 24*, 3107–3113.
Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.
Vanderwerf, P., & Ullman, E. F. (1980) *Biochim. Biophys. Acta 596*, 302–314.
White, J., Kielian, M., & Helenius, A. (1983) *Q. Rev. Biophys. 16*, 151–195.
Wilschut, J., Düzgünes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.
Wilson, I., Skehel, J., & Wiley, D. (1981) *Nature (London) 289*, 366–373.

Absence of Detectable Ribonucleic Acid in the Purified, Untransformed Mouse Glucocorticoid Receptor†

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ABSTRACT: The glucocorticoid receptor (GC-R) isolated from the mouse AtT-20 pituitary tumor cell line exists in three forms. The untransformed (non-DNA-binding), 9.1S species (319K) can be converted into two transformed (DNA-binding) species. One of these (5.2 S, M_r 132K) appears to be composed of one molecule of the hormone-binding, monomeric protein (96K) plus a small RNA, while the second transformed species is the monomeric, hormone-binding subunit (3.8 S, 96K) itself. We wished to determine whether the untransformed GC-R contains RNA or if the monomer binds to RNA subsequent to subunit dissociation (which occurs during receptor transformation). Kinetic studies using both the crude and purified untransformed GC-R show that the untransformed, 9.1S GC-R dissociates into 3.8S monomeric subunits, without forming a transient 5.2S complex. The untransformed receptor was then purified with affinity chromatography, gel filtration, and DEAE-cellulose chromatography. One major protein band, corresponding in size to the GC-R monomer (94K–96K), was observed on sodium dodecyl sulfate–polyacrylamide gels upon silver staining or fluorography of [^{3}H]dexamethasone mesylate covalently labeled receptor. In vivo ^{32}P-labeling of AtT-20 cells, followed by purification of the untransformed GC-R, yielded two major ^{32}P-labeled components (94K–96K and 24K). Both of these bands were protease-sensitive, contained phosphoserine, and were unaffected by ribonuclease treatment. We conclude that the untransformed mouse GC-R is wholly proteinaceous and contains no RNA. Thus, RNA binding occurs subsequent to dissociation of the oligomeric, untransformed GC-R complex into monomers.

Steroid hormone receptors are gene regulatory proteins that elicit a wide variety of physiological responses (Yamamoto & Alberts, 1976). Crucial to our understanding of the mechanism of steroid-regulated gene expression is a knowledge of the structure of these proteins. A large number of studies support the hypothesis that an oligomeric, untransformed (non-DNA-binding) receptor species dissociates into subunits upon receptor transformation, that is, conversion to a DNA-binding moiety [reviewed in Vedeckis (1985)]. Our laboratory has performed a detailed series of studies to elucidate the structure of the glucocorticoid receptor (GC-R)[1] in the mouse AtT-20 pituitary tumor cell line (Vedeckis, 1981, 1983a,b, 1985; Eastman-Reks et al., 1984; Reker et al., 1985; Kovačič-Milivojević et al., 1985; Vedeckis et al., 1985). We

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[1] Abbreviations: 94K, 24K, etc., M_r of 94 000, 24 000, etc.; BSA, bovine serum albumin; DEAE, diethylaminoethyl; Dex-M, dexamethasone 21-mesylate (9α-fluoro-16β-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 21-methanesulfonate); EDTA, ethylenediaminetetraacetic acid; GC-R, glucocorticoid receptor; HPLC, high-performance liquid chromatography; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TA, triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetal with acetone); TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; UMP, uridine 5′-phosphate.

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have found that the untransformed GC-R is an oligomeric protein (R_s = 8.3 nm, 9.1 S, M_r 319K). Upon receptor transformation, two transformed species are generated. One of these is the monomeric, hormone-binding protein subunit (6 nm, 3.8 S, M_r 96K). Our recent studies have concentrated on a transformed GC-R of intermediate size (6 nm, 5.2 S, M_r 132K). Currently, we have substantial evidence (Kovačič-Milivojević et al., 1985) indicating that this oligomeric GC-R species contains one molecule of the hormone-binding, monomeric protein (M_r 96K) plus a small RNA species (M_r 24K–36K).

This study was undertaken to determine if RNA is also a component of the native, untransformed GC-R oligomer. This was essential in order to elucidate what physiological significance the receptor–RNA interaction may have. For example, if RNA were necessary for the structural integrity of the untransformed complex, it would be expected to be an integral component of this receptor form. Alternatively, if the interaction with RNA represented a possible feedback mechanism for hormone-regulated gene expression or a mechanism for posttranscriptional control of gene expression, one would expect that the untransformed receptor would not contain RNA. Rather, it would be likely that the receptor monomer formed after subunit dissociation would subsequently bind to RNA. This study distinguishes between these possibilities and demonstrates that RNA is absent from the oligomeric, untransformed GC-R complex.

Materials and Methods

Cell Culture, Labeling Conditions, and Cytosol Preparation. The mouse AtT-20 pituitary tumor cell line was maintained in T flasks and spinner suspension culture as described previously (Vedeckis, 1981). When not used for purification, GC-R prepared in TETg (20 mM Tris-HCl, 1 mM EDTA, 12 mM 1-thioglycerol, pH 7.4 at 22 °C) buffer was labeled with 3×10^{-8} M [^{3}H]TA overnight at 0–4 °C. For ^{32}P-labeling experiments, cells in log-phase growth were pelleted from 2 L of culture and washed with MEM/Joklik phosphate-free medium containing 5% dialyzed calf serum. The cellular pellet (3–4 mL) was then suspended in 300 mL of this media containing [^{32}P]orthophosphate (40–60 μCi/mL, ICN Biomedicals, Inc.) at 37 °C. Following an initial 3–4-h incubation, cells were diluted to 3 L with the same medium and incubated for an additional 14–16 h. All subsequent steps were done at 0–4 °C. Cells were harvested and resuspended in 1 volume of TMTg buffer (20 mM Tris-HCl, 10 mM sodium molybdate, 10 mM 1-thioglycerol, pH 7.0 at 22 °C) and ruptured by stainless steel Dounce (Kontes) homogenization. The homogenate was centrifuged at 5000g for 10 min. The upper lipid layer was aspirated carefully and the supernatant fraction centrifuged at 190000g_{av} for 1 h to obtain cytosol.

Purification of Untransformed Glucocorticoid Receptor. Untransformed GC-R was purified as described by Grandics et al. (1984a) with slight modifications. Cytosol (3–6 mL) prepared in TMTg buffer was immediately mixed with 3–4 mL of Sterogel A affinity resin and incubated for 2 h at 0 °C with gentle stirring. After incubation the resin was packed into a column and washed extensively with 125 mL of TMTg buffer, followed by 50 mL of TMTgG buffer (TMTg buffer containing 10% glycerol). The adsorbed receptor was then eluted from the resin by suspension in an equal volume of TMTgG buffer containing 2 μM [^{3}H]TA (41.8 Ci/mmol, New England Nuclear) for 14–16 h.

In some experiments the receptor was eluted from the resin with [^{3}H]dexamethasone 21-mesylate (Dex-M, 48.9 Ci/mmol, New England Nuclear). In those cases, the affinity resin was washed with TMG buffer (TMTgG without 1-thioglycerol, adjusted to pH 8), and the GC-R was eluted from the resin by suspension in a half-volume of TMG buffer containing 0.5 μM [^{3}H]Dex-M. After a 4-h incubation with gentle stirring, 1-thioglycerol was added to a final concentration of 10 mM. In order to elute all the GC-R possible, a half-volume of TMG buffer containing 2 μM [^{3}H]TA was added, followed by incubation for an additional 12 h. The slurry was centrifuged, the supernatant was collected, and the resin was washed with 1 volume of TMG buffer to ensure recovery of a majority of the receptor. The affinity resin eluate was immediately chromatographed in TMTg buffer on a column (2.6 × 60 cm) of Agarose A-1.5m, 200–400 mesh (Bio-Rad), at a flow rate of 27 mL/h. Three-milliliter fractions were collected, and aliquots (0.3 mL) were taken for counting. The receptor peak fractions were pooled and applied to a 2-mL DEAE-cellulose column equilibrated in TMTg buffer. The column was washed with 10 volumes of TMTg buffer, and the GC-R was eluted with a salt gradient of 0–0.5 M KCl in TMTg buffer at flow rate 50 mL/h. The peak fractions containing the receptor-associated radioactivity were immediately further analyzed.

High-Performance Liquid Chromatography (HPLC). Protein fractionation and characterization were carried out by HPLC on an Altex TSK-3000SW exclusion column (7.5 × 300 mm) preceded by a TSK-3000SW guard column (7.5 × 100 mm) as described elsewhere (LaPointe et al., 1986). To determine the Stokes radius (R_s) of the GC-R, the column was calibrated with ^{14}C-methylated RNase A (1.64 nm), ovalbumin (2.86 nm), bovine serum albumin (3.59 nm), ferritin (6.15 nm), and thyroglobulin (8.61 nm). The void volume (V_0) and total volume (V_t) were determined spectrophotometrically or by conductivity measurement with blue dextran and 3 M KCl, respectively.

Sucrose Gradient Analysis. Vertical tube rotor (VTi 80) ultracentrifugation was carried out as described previously (Reker et al., 1985). Five to twenty percent sucrose gradients (5.2 mL) were prepared in TETg or TMTg buffer. Sedimentation properties of GC-R complexes were analyzed with ^{14}C-methylated standards run in parallel tubes (Eastman-Reks et al., 1984). The untransformed GC-R sediments at 9.1 S. The monomeric, transformed and oligomeric, transformed GC-R species sediment at 3.8 and 5.2 S, respectively, on molybdate-containing, low-salt sucrose gradients. In molybdate-free, low-salt sucrose gradients the monomeric and oligomeric transformed species sediment somewhat faster, at 4.2 and 6.6 S, respectively.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Proteins were electrophoresed in 10% polyacrylamide gels overlayed with a 4% polyacrylamide stacking gel (Laemmli, 1970). Receptor samples were concentrated by precipitation with 10% trichloroacetic acid (TCA) together with 0.02% deoxycholate at 0 °C for 30 min. The pellets were washed once with ice-cold 10% TCA, twice with an ice-cold mixture of ether/acetone (4:7), and then redissolved in SDS sample buffer. To ensure complete solubilization of acid-precipitated material, the samples were briefly sonicated prior to boiling at 100 °C for 5 min. Electrophoresis was performed at 15-mA constant current until the tracking dye reached the separating gel, and then the current was increased to 30 mA. Total run time was about 4.5 h. Gels were fixed in 50% methanol for 60 min and then stained with a silver stain technique (Wray et al., 1981). In some experiments the fixed gel was washed with water, dried, and subjected to autoradiography at −70 °C with XRP-5 X-ray film (Kodak) and Du Pont intensifying screens for 2–3 days. For ^{3}H-labeled proteins, gels were im-

pregnated with Fluorohance (Research Products International Corp.) according to the manufacturer's directions before drying. In the experiment using [^{3}H]Dex-M-labeled receptor, the gel system of Dreyfuss et al. (1984) was used.

Phosphoamino Acid Analysis. ^{32}P-Labeled proteins were eluted from gels by electrophoresis into dialysis tubing exactly as described by Clinton et al. (1979) and concentrated by precipitation with 10% TCA. Pellets were washed twice with ice-cold acetone, suspended in 6 N HCl, and hydrolyzed at 110 °C for 1 h. The hydrolyzed samples were dried in vacuo and suspended in 10 μL of water containing 1 mg/mL each of phosphoserine, phosphothreonine, and phosphotyrosine as markers. The samples were spotted onto Whatman 3MM paper and electrophoresed in a buffer at pH 3.5 containing 5% glacial acetic acid and 0.5% pyridine. Electrophoresis was at 2400 V for 1 h. After electrophoresis the dried paper was autoradiographed with intensifying screens at −70 °C for 10 days.

RESULTS

HPLC Transformation of GC-R. It has been shown that the oligomeric, transformed receptor is composed of the monomeric hormone-binding protein subunit (M_r 96K) and a low molecular weight RNA (M_r 20K–40K) (Kovačič-Milivojević et al., 1985). With reference to the formation of the RNA-containing oligomeric (5.2–6.6 S) GC-R, two possible mechanisms have been proposed. First, RNA-containing 9.1S, untransformed complexes could dissociate into RNA-containing 5.2S–6.6S complexes. Second, non-RNA-containing 9.1S, untransformed oligomers could dissociate into 3.8S–4.2S monomers. The monomers could then subsequently bind to low molecular weight RNA molecules present in the cytosolic extract. To distinguish between these possibilities, two basic approaches have been employed in this study: (1) the kinetics of GC-R transformation were evaluated followed by an analysis of the nature of the resultant transformed species, and (2) a biochemical analysis of the *purified* 9.1S, untransformed GC-R was performed.

The most common method of GC-R transformation we use is Sephadex G-25 gel filtration. However, under these conditions most macromolecules (including RNA) coelute with the GC-R. With this in mind we used HPLC to not only promote GC-R transformation but also to separate low molecular weight RNA molecules from the GC-R complex. [^{3}H]TA-Labeled crude cytosol (250 μL) was subjected to HPLC, and the peak fraction containing the 8.3-nm GC-R (which corresponds to 9.1S, untransformed GC-R) was then allowed to transform spontaneously at 0–4 °C for different time periods (30 and 120 min). At the end of the incubation, 20 mM sodium molybdate was added to prevent further transformation. Each sample was then analyzed on molybdate-free, low-salt sucrose gradients. As can be seen from Figure 1, after HPLC the GC-R sediments as the untransformed 9.1S form (zero time point), which rapidly undergoes transformation to generate a sharp 4S peak. This result suggests that RNA is not an integral component of the untransformed, oligomeric 9.1S GC-R but, rather that a wholly proteinaceous 9.1S, untransformed GC-R dissociates into the 3.8S–4.2S transformed monomer. Since low molecular weight RNA molecules are separated from the GC-R during HPLC, the subsequent formation of the oligomeric, 5.2S–6.6S species cannot occur.

Purification of Untransformed GC-R. We used a recently developed technique for purification of the untransformed GC-R (Grandics et al., 1984a). AtT-20 cell GC-R was purified by a three-step procedure utilizing affinity chromatography, agarose gel filtration, and DEAE-cellulose chromatography. We routinely obtained about a 3000–4000-fold purification of the GC-R, with about a 30% yield (data not shown). The purity of the receptor was estimated to be 75–90%. During purification in the presence of 10 mM sodium molybdate, the GC-R retains the same characteristics as the cytosolic GC-R; namely, it elutes from an agarose A-1.5 m column with a Stokes radius of 8.3 nm (see below). Additionally, the GC-R elutes from the DEAE-cellulose column as a single peak at 0.18 M KCl. This elution position corresponds to that of the cytosolic, untransformed GC-R (Vedeckis, 1983b; Reker et al., 1985). Receptor-containing peak fractions eluted from DEAE-cellulose (DEAE-cellulose pool) were subjected to HPLC. A single sharp peak with a Stokes radius of 8.2 nm was obtained (data not shown).

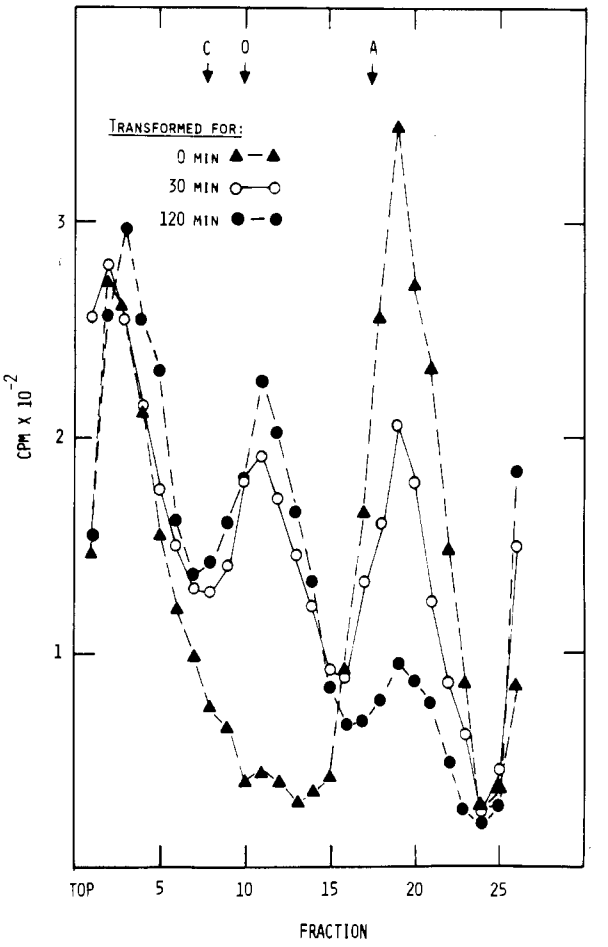

FIGURE 1: HPLC transformation of glucocorticoid receptor. Cytosol labeled with [^{3}H]TA was subjected to HPLC with 50 mM phosphate buffer. To promote receptor transformation, the HPLC peak fraction (R_s = 8.2 nm) was incubated at 0–4 °C for 0 (▲), 30 (○), and 120 (●) min. (For each time point a separate 250-μL aliquot of cytosol was subjected to HPLC.) At the end of the incubation, 20 mM sodium molybdate was added to the peak fraction containing GC-R. For the zero time point, 20 mM sodium molybdate was added to each collection tube directly, before we started to collect fractions from HPLC. Two hundred microliters of each sample was analyzed on 5–20% sucrose gradients (prepared in TETg buffer) at 2 °C in a vertical tube rotor. The arrows designate the sedimentation positions of standard proteins run on parallel gradients: chymotrypsinogen A (C, 2.6 S), ovalbumin (O, 3.5 S), and aldolase (A, 7.9 S).

Sucrose gradient analysis of the same GC-R preparation demonstrated a sharp peak in the 9.2S region of the gradient (Figure 2A). RNase A treatment (90 units/mL, 60 min at 0 °C) of this preparation had no effect on the sedimentation value of untransformed GC-R (Figure 2A). Direct evidence that the final receptor preparation contains untransformed

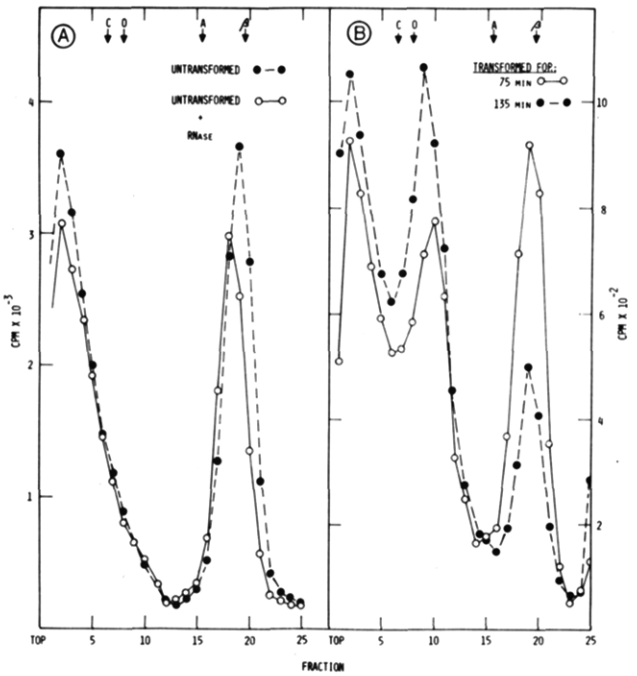

FIGURE 2: Transformation of purified glucocorticoid receptor. A total of 3 mL of the purified, untransformed GC-R-containing peak fractions from DEAE-cellulose chromatography was pooled. (Panel A) A total of 0.3 mL of the DEAE-cellulose pool was treated with RNase A (90 units/mL for 60 min at 0 °C) (○). Another identical sample was kept on ice (●). Two hundred microliters of each sample was then analyzed on a 5–20% sucrose gradient prepared in TMTg buffer. (Panel B) A total of 2.4 mL of the DEAE-cellulose pool was applied to a 6-mL (packed volume) Sephadex G-25 column equilibrated in 20 mM Tris-HCl, 10 mM 1-thioglycerol, and 100 μg/mL BSA buffer (pH 7 at 22 °C) at 0–4 °C. One-milliliter fractions were collected with the same buffer. The excluded peak fraction containing GC-R, monitored by radioactivity, was divided into two portions and incubated on ice for 75 (○) or 135 min (●). A total of 200 μL of each sample was layered on the same type of sucrose gradient as in panel A. Standard proteins and conditions are the same as indicated in the legend to Figure 1, with the addition of β-amylase (β, 9.4 S) as an additional standard.

GC-R similar to that found in cytosol is the ability to transform the GC-R preparation. In order to remove molybdate and high salt, the DEAE-cellulose pool was filtered through a Sephadex G-25 column equilibrated in TTgB buffer (20 mM Tris-HCl, 10 mM 1-thioglycerol, 100 μg/mL BSA, pH 7 at 22 °C) and incubated at 0–4 °C to allow for GC-R transformation to occur. After 75 or 135 min, sodium molybdate was added to each sample (20 mM final concentration) to prevent further transformation. Analysis of this material by sucrose gradient ultracentrifugation demonstrated a 9.1S, oligomeric untransformed receptor plus transformed receptor in the 4S region of the sucrose gradient. After 75 min of incubation about 40% of the bound radioactivity corresponds to the transformed 4S GC-R (Figure 2B), while 70% of the bound radioactivity was found in the 4S region of the sucrose gradient after a further 60-min incubation (Figure 2B). Thus, the purified, untransformed GC-R has properties similar to those of the untransformed GC-R in crude cytosol.

Sephadex G-25 gel filtration of crude cytosol always results in the formation of the 5.2S–6.6S, oligomeric, transformed species (Reker et al., 1985; Kovačič-Milivojević et al., 1985). These experiments with purified untransformed GC-R resulted in the generation of only the 4S receptor, similar to the data generated by HPLC transformation. The most likely explanation for these results is that both HPLC and purification of the untransformed GC-R result in the removal of excess, free RNA molecules from the receptor preparation. Thus,

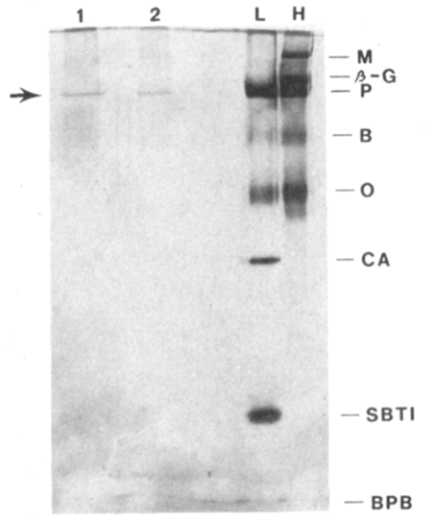

FIGURE 3: Silver-stained gel of purified, untransformed glucocorticoid receptor. The untransformed mouse AtT-20 cell glucocorticoid receptor was purified as described under Materials and Methods. One milliliter (lane 1) and 0.75 mL (lane 2) of the DEAE-cellulose pool material were precipitated with TCA and subjected to SDS–PAGE, followed by silver staining of the gel. The arrow points to the 95K silver-stained band. Low (L) and high (H) molecular weight standards (Bio-Rad) were also run. The molecular weights of these standards are as follows: myosin (M, 200K); β-galactosidase (β-G, 116.2K); phosphorylase B (P, 97.5K); bovine serum albumin (B, 66.2K); ovalbumin (O, 45K); carbonic anhydrase (CA, 31K); soybean trypsin inhibitor (SBTI, 21.5K). BPB is the bromophenol blue dye front.

formation of the 4S monomer, instead of the 5.2S–6.6S, RNA-containing, oligomeric transformed species, is evidence that RNA is not an integral component of the 9.1S, oligomeric untransformed GC-R.

Characterization of Purified Untransformed GC-R. The purified GC-R preparation was routinely analyzed by SDS–PAGE to determine the size of the purified subunits present in the untransformed complex and the purity of the DEAE-cellulose pool. The protein pattern (Figure 3) showed a major protein band that migrated slightly faster than the phosphorylase B marker protein, having a M_r of 95 000 (±2000, n = 6). A few additional faint bands usually could be seen with a molecular weight higher than that of phosphorylase B as well as in the 60K–70K region of the gel.

To determine if the 95K band obtained by SDS–PAGE analysis might represent the authentic GC-R monomer, we eluted the GC-R from the affinity column with [^{3}H]dexamethasone 21-mesylate ([^{3}H]Dex-M). As shown in Figure 4, when the affinity matrix was eluted with 0.5 μM [^{3}H]-Dex-M (lane 1) and subsequently chromatographed through Bio-Gel A-1.5m (lane 2) and then DEAE-cellulose (lane 4), one major band (M_r 96K) could be identified on the fluorogram. In addition, macromolecules of 81K, 57K, 44K, and 38K bound [^{3}H]Dex-M and could represent proteolytic fragments of the 96K protein, or minor contaminants. In order to determine if RNA may be covalently attached to the [^{3}H]Dex-M-labeled proteins and to test if contaminating proteases were present in our ribonuclease preparation, we treated an aliquot of the [^{3}H]Dex-M-labeled, purified, untransformed receptor with 90 units/mL of RNase A for 60 min at 0 °C before TCA precipitation. No effect of this treatment was noted (Figure 4, lane 3).

Finally, it is possible to "zero-distance" covalently cross-link ribonucleoproteins with high-intensity UV irradiation (Mayrand & Pederson, 1981). Thus, we irradiated an aliquot of the DEAE-cellulose pool at 254 nm at 4000 μW/cm^2 for 10 min, prior to TCA precipitation (Figure 4, lane 5). We could

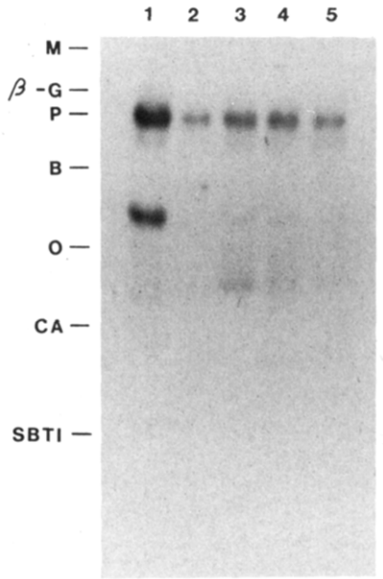

FIGURE 4: Fluorography of purified untransformed glucocorticoid receptor labeled with [^{3}H]dexamethasone 21-mesylate and run on SDS gels. AtT-20 cell cytosol was incubated with an affinity resin and then eluted with [^{3}H]Dex-M and [^{3}H]TA as described under Materials and Methods. The affinity eluate (lane 1) was sequentially purified on an agarose A-1.5m column (lane 2) and then on DEAE-cellulose (lanes 3–5). Aliquots of the purified, untransformed GC-R (DEAE-cellulose pool) were treated with 90 units/mL RNase A at 0 °C for 1 h (lane 3), left untreated on ice (lane 4), or irradiated at 254 nm with 4000 μW/cm^2 for 10 min (lane 5). All samples were precipitated with TCA and submitted to SDS–PAGE, according to the method of Dreyfuss et al. (1984). Standard proteins were run on an adjacent lane that was cut from the gel and stained. The standards are the same as those described in the legend to Figure 3.

detect no increase in the molecular weight of the [^{3}H]Dex-M-labeled 96K band, suggesting again that RNA was not present in the purified, untransformed GC-R preparation.

Purification of Untransformed GC-R from Cells Grown in [^{32}P]Orthophosphate. It has been shown that both the glucocorticoid and progesterone receptor proteins are phosphorylated (Housley & Pratt, 1983, 1984; Grandics et al., 1984b; Singh & Moudgil, 1985; Dougherty et al., 1982; Puri & Toft, 1984). Other laboratories have also found a low molecular weight macromolecule (21K–24K), which can be labeled in vivo with ^{32}P, copurifying with the GC-R. Some evidence suggests this macromolecule is RNA (Grandics et al., 1984b; Housley & Pratt, 1984). Since we were not able to detect this band by silver staining, we attempted to purify the untransformed GC-R from cells labeled in vivo with ^{32}P and to look for a low molecular weight band that could be a small RNA molecule. Cells were incubated with [^{32}P]orthophosphate in order to label GC-R in vivo, and the untransformed receptor was then subjected to the three-step purification protocol. ^{32}P-Containing material coeluted with the peak of [^{3}H]TA during gel filtration on the agarose A-1.5m column (Figure 5A) and during the final DEAE-cellulose chromatography step (Figure 5B).

The various steps of the GC-R purification scheme were then analyzed by autoradiography of the ^{32}P-labeled material (Figure 6). The relative intensities of the various bands in different steps of the purification cannot be directly compared as the amount of protein added to each lane is not identical. The predominant ^{32}P-labeled band is a 95K moiety, identical with that shown on the silver-stained gel in Figure 3 and the [^{3}H]Dex-M-labeled band in Figure 4. Of interest is the 24K component, which was not detected by silver staining. This 24K macromolecule could be a proteolytic product of the 95K protein, a contaminating protein, or a small polynucleotide, as has been suggested by Grandics et al. (1984b). An additional very faint band, with an apparent M_r of 84K, could be seen. Thus, our data using untransformed, purified ^{32}P-labeled GC-R are in accord with those reported by Housley and Pratt (1983, 1984) in L cells and Grandics et al. (1984b) and Singh and Moudgil (1985) in rat liver.

We next determined the nature of the 95K, 84K, and 24K bands using different enzymatic treatments. When the DEAE pool was treated with alkaline phosphatase, only the very faint 84K band was affected as determined by autoradiography (Figure 6A, lane 5). As is also shown in Figure 6A, all three ^{32}P-labeled bands were eliminated upon digestion with proteinase K (lane 6) and trypsin (lane 7). In another experiment, a 10 times lower concentration of trypsin was used to digest the DEAE pool (Figure 6B, lane 1), and extensive fragmentation of the ^{32}P-labeled 95K protein was seen. Discrete fragments with apparent M_r of 43K, 38.5K, and 10K were detectable. The 59K and 28.5K fragments were also seen upon lengthy exposure of this same gel (data not shown). Since these sizes correspond quite well to authentic GC-R fragments observed previously [reviewed in Vedeckis (1985), Vedeckis et al. (1985), and Reichman et al. (1984)], this suggests (but does not prove) that the 95K, ^{32}P-labeled band represents the GC-R protein itself and not some contaminating phosphoprotein copurifying with the receptor. When the DEAE pool was treated with pancreatic RNase A (50 units/mL in Figure 6A, lane 8, and 100 units/mL in Figure 6B, lane 2), no substantial difference was seen in the size or intensity of labeling of the 95K moiety. Also, the 24K band was not digested by RNase A treatment. However, the 24K band did appear more diffuse after RNase A treatment, and several faint bands were seen migrating between the bovine serum albumin and ovalbumin standards, even when soybean trypsin inhibitor was present during the digestion. We attribute this to some non-trypsin-like protease contamination still active in the ribonuclease enzyme preparation. When RNase T_1 (100 units/mL) was added, the autoradiogram showed the same pattern as the control sample (compare Figure 6A, lane 9, and Figure 6B, lane 3, with Figure 6A, lane 3). If the ^{32}P-labeled DEAE-cellulose pool was extracted with phenol/chloroform and the aqueous phase analyzed for RNA content on a 7 M urea gel, no RNA was detected (data not shown). Proteins were also precipitated from the phenol phase with TCA and analyzed by SDS–PAGE. Both the 95K and 24K bands were recovered (data not shown). Thus, these results and those presented in Figure 6 show that the 24K, ^{32}P-labeled band that copurifies with the untransformed GC-R is a protein and is not RNA.

Phosphoamino Acid Analysis. To further analyze the nature of the ^{32}P-labeled 95K and 24K bands, phosphoamino acid analysis was performed. Figure 7A shows the SDS–PAGE of the ^{32}P-labeled, purified untransformed receptor preparation. As was shown in Figure 6, two phosphorylated protein bands with M_r of 94K and 24K were detected. The phosphoamino acid content of the 94K and 24K phosphorylated proteins was examined by excising the bands from the gel (Figure 7A), followed by phosphamino acid analysis. The only phosphoamino acid detected in both bands (94K and 24K) was phosphoserine (Figure 7B).

These results with the 94K protein are consistent with previous observations that the progesterone (Dougherty et al., 1982) and glucocorticoid (Housley & Pratt, 1983) receptors are phosphorylated on serine residues, although others claim that the GC-R contains phosphothreonine (Miller-Diener et

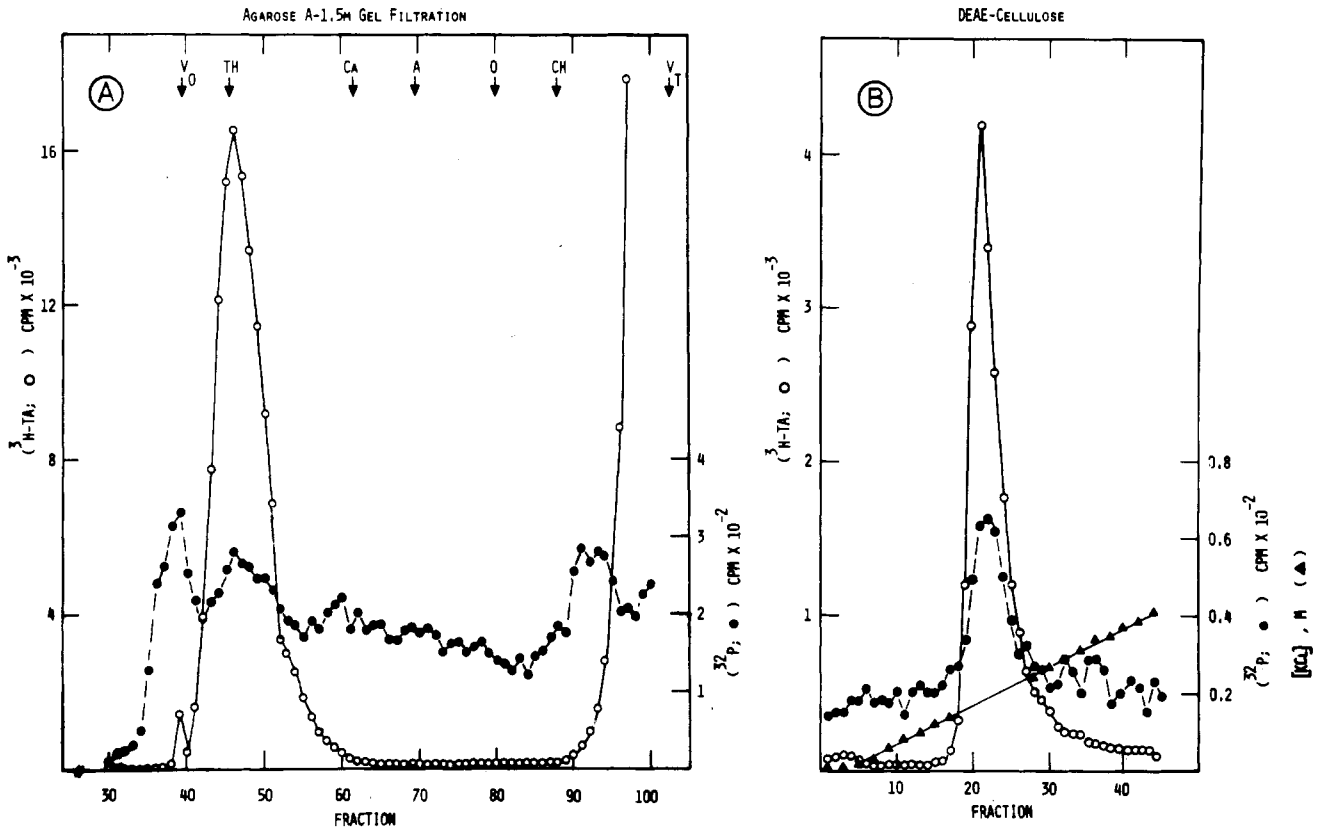

FIGURE 5: Purification of in vivo ^{32}P-labeled, untransformed glucocorticoid receptor complex. Cytosol was prepared from in vivo ^{32}P-labeled AtT-20 cells, and the GC-R was purified as described under Materials and Methods with ^{3}H[TA] to elute the receptor from the affinity resin. (Panel A) The affinity eluate was chromatographed on an agarose A-1.5m column equilibrated in TMTg buffer. Fractions (3 mL) were collected, and 0.3-mL aliquots were taken for counting radioactivity. Fractions 43–51 were pooled and applied to a 2-mL (packed volume) DEAE-cellulose column (Panel B). The GC-R was eluted with a 0–0.5 M KCl gradient. One-milliliter fractions were collected, and 50-μL aliquots were taken for the measurement of radioactivity. The conductivity (▲) was measured in each fraction with a Radiometer conductivity meter: (○) ^{3}H[TA] bound to the GC-R; (●) ^{32}P-labeled molecules.

al., 1985). Our results also indicate that the 24K band contains phosphoserine and is, therefore, a protein. Of particular significance is the absence of any ^{32}P-labeled material migrating with the phosphotyrosine standard. In this one-dimensional system, UMP comigrates with phosphotyrosine (Clinton & Huang, 1981). Thus, these studies show conclusively that the 94K–96K and 24K ^{32}P-labeled materials isolated during purification of the untransformed GC-R are, indeed, proteins. We thus conclude that the untransformed GC-R is wholly proteinaceous and that RNA is not an integral component of this complex.

Discussion

The finding that the mouse GC-R can exist as a ribonucleoprotein (Reker et al., 1985; Kovačič-Milivojević et al., 1985) raised some important questions, the most critical of which was whether the oligomeric, untransformed GC-R (9.1 S, M_r 319K) also contains bound RNA. Thus, the major goal of this study was to determine if RNA is an integral component of the untransformed GC-R complex.

The kinetic analyses of GC-R transformation we present here were performed in a manner to eliminate the presence of excess free, small RNA molecules (HPLC; use of the purified, untransformed GC-R). This showed that the 9.1S, untransformed GC-R was converted directly into 3.8S monomers, with no 5.2S intermediate GC-R being formed. This is in contrast to our earlier studies using crude cytosolic GC-R transformed by G-25 filtration, in which the 5.2S GC-R was obtained. Thus, we believe that when crude cytosol is used, the 9.1S, untransformed GC-R also dissociates into 3.8S monomers. However, the monomers then apparently bind rapidly and quantitatively to small RNA molecules present in the crude cytosol, yielding the 5.2S form.

More definitive answers about the structure of the untransformed GC-R were obtained by purifying this species. Silver-stained SDS gels of the purified receptor showed a major band with an apparent M_r of 94K–96K. This is identical with the molecular weight of the authentic, hormone-binding, monomeric, GC-R subunit, as determined by purifying the untransformed GC-R that was affinity-labeled with [^{3}H]-Dex-M. Finally, after in vivo labeling of intact cells with [^{32}P]orthophosphate, only two major ^{32}P-labeled bands were seen in the purified, untransformed GC-R preparation. One of these (94K–96K) was identical in molecular weight with the hormone-binding monomer, while the second (24K) was not detected on the silver-stained gels. Enzymatic treatment of the purified preparations showed that both the 94K–96K and 24K species were proteinaceous. The demonstration that both species contained phosphoserine virtually eliminates the possibility that these ^{32}P-labeled bands contain RNA. The only way RNA might still be present in the untransformed GC-R complex is if does not stain with the silver stain and turns over so extraordinarily slowly that it was not labeled in the 18-h incubation period. We believe this to be an extremely unlikely possibility.

Although the major goal of this study was not to determine the actual stoichiometry of the protein subunits in the oligomeric, untransformed GC-R complex, recent reports have suggested that a 90K, non-hormone-binding, phosphoprotein is a common subunit of untransformed glucocorticoid, progesterone, estrogen, and androgen receptors (Birnbaumer et al., 1984; Dougherty et al., 1984; Joab et al., 1984; Renoir et

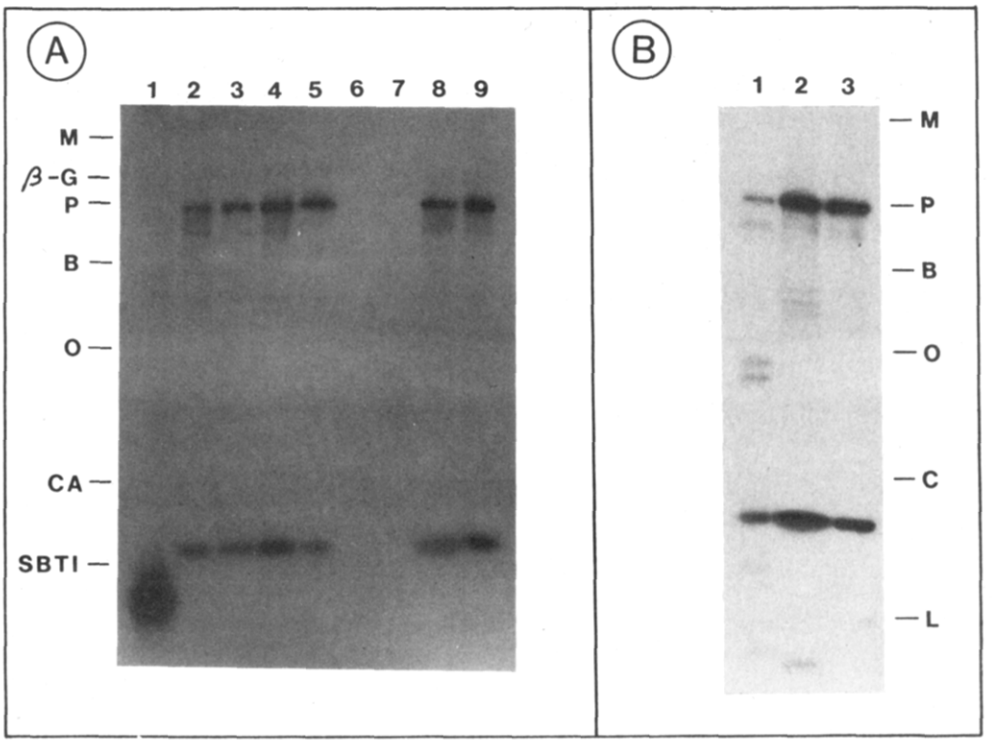

FIGURE 6: Autoradiography of purified, ^{32}P-labeled, untransformed glucocorticoid receptor run on SDS–PAGE. (Panel A) Aliquots from various stages of purification (140 μL for lane 1, 1.5 mL for lane 2, and 1 mL for lanes 3–9) were TCA-precipitated and analyzed as described under Materials and Methods. (Lane 1) Affinity gel eluate; (lane 2) agarose A-1.5m pool; (lanes 3–9) DEAE-cellulose pool (fractions 19–25 in Figure 5B, pooled together). These samples were incubated with various enzymes for 30 min at 37 °C, prior to TCA precipitation. (Lane 3) The 0 °C control without additions; (lane 4) 37 °C control without additions; (lane 5) plus alkaline phosphatase (100 units/mL); (lane 6) plus proteinase K (50 μg/mL); (lane 7) plus trypsin (50 μg/mL); (lane 8) plus RNase A (50 units/mL); (lane 9) plus RNase T_1 (100 units/mL). (Panel B) Protease and RNase sensitivity of ^{32}P-labeled purified GC-R obtained in a separate experiment. The same amount of DEAE-cellulose pool as in panel A was treated in the same way with trypsin (5 μg/mL) (lane 1), RNase A (100 units/mL) in presence of SBTI (500 μg/mL) (lane 2), and RNase T_1 (100 units/mL) (lane 3). The molecular weight markers are the same as described in the legend to Figure 3 with the addition of chymotrypsinogen A (C, 25.7K) and lysozyme (L, 14.4K).

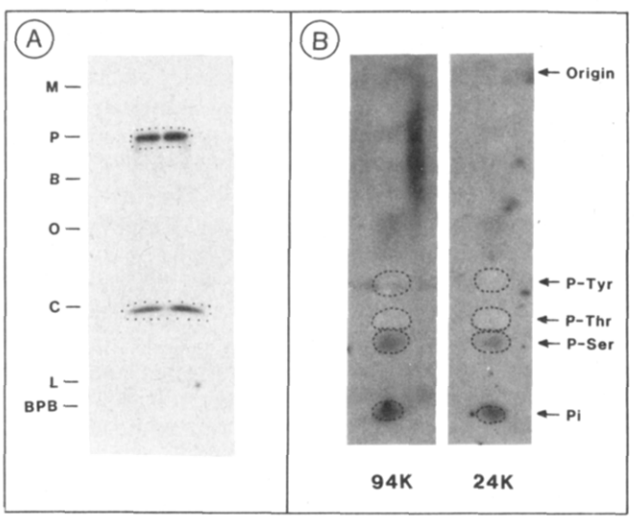

FIGURE 7: Phosphoamino acid analysis of purified, untransformed glucocorticoid receptor. (Panel A) The 95K and 24K proteins from the ^{32}P-labeled, purified, untransformed GC-R were excised and eluted from the gel by electrophoresis. (Panel B) The proteins were partially acid hydrolyzed and analyzed by high-voltage paper electrophoresis in one dimension. The ^{32}P-labeled phosphoamino acids were detected by autoradiography, and the markers were localized by ninhydrin. Standard markers used were as follows: P-Tyr, phosphotyrosine; P-Thr, phosphothreonine; P-Ser, phosphoserine. P_i represents inorganic phosphate.

al., 1984; Puri & Toft, 1984; Sullivan et al., 1985). This 90K protein is apparently phosphorylated on serine (Dougherty et al., 1982) and may be a heat shock protein (Sanchez et al., 1985). We have not observed a 90K protein band upon silver staining of our purified GC-R complex run on SDS gels. Furthermore, only one ^{32}P-labeled (at serine) band is seen in the 94K–96K region of the gel, identical in size with that of [^{3}H]Dex-M-labeled, authentic, GC-R monomeric hormone-binding subunit. There are three possible explanations for this. First, in our system (10% PAGE), it is possible that the "90K" protein migrates with a higher molecular weight (94K–96K), absolutely coincident with the GC-R monomer. Second, the minor 84K ^{32}P-labeled band (Figure 6) might be the 90K protein. The absence of this 84K band in some preparations (e.g., Figure 7A) makes it less likely that this protein is an integral component of the untransformed GC-R complex. Alternatively, it is possible that the 9.1S, oligomeric, untransformed GC-R purified from AtT-20 cells does not contain any 90K protein. This would be a significant finding that needs to be analyzed by immunochemical techniques. We are currently performing these studies.

Finally, we do not yet know whether the 24K phosphoprotein we observe is a separate subunit in the untransformed GC-R complex or a proteolytic fragment of the GC-R monomer. The occasional presence of 70K silver-staining bands raises the possibility that the 24K (non-silver-staining) and 70K "contaminants" we observe may be the products of 94K–96K GC-R monomer proteolysis. Further, the relative amounts of ^{32}P-labeled 94K–96K and 24K bands are not constant among preparations. This lack of stoichiometry makes receptor fragmentation a more likely possibility than the 24K moiety being a separate subunit of the untransformed complex. Proteolyzed GC-R molecules can be purified as an intact untransformed complex when molybdate is present (Mendel et al., 1985).

To summarize, the 9.1S, untransformed GC-R from mouse AtT-20 cells is wholly proteinaceous and does not appear to contain RNA. This suggests that the untransformed GC-R is transformed directly into 3.8S, monomeric subunits. The 3.8S, monomeric GC-R subunits then bind to a low molecular weight RNA species. Recent studies in our laboratory indicate that this RNA species contains a mixture of various tRNA molecules (M. Ali and W. V. Vedeckis, unpublished data). Further studies are needed to determine if the interaction of the GC-R monomer with these tRNA species is of a specific nature and if it is physiologically relevant, for example, in posttranscriptional processes.

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REFERENCES

Birnbaumer, M., Bell, R. C., Schrader, W. T., & O'Malley, B. W. (1984) *J. Biol. Chem. 259*, 1091–1098.

Clinton, G. M., & Huang, A. S. (1981) *Virology 108*, 510–514.

Clinton, G. M., Burge, B. W., & Huang, A. S. (1979) *Virology 99*, 84–94.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 14226–14230.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1984) *J. Biol. Chem. 259*, 8004–8009.

Dreyfuss, G., Adam, S. A., & Choi, Y. D. (1984) *Mol. Cell. Biol. 4*, 415–423.

Eastman-Reks, S. B., Reker, C. E., & Vedeckis, W. V. (1984) *Arch. Biochem. Biophys. 230*, 274–284.

Grandics, P., Miller, A., Schmidt, T. J., Mittman, D., & Litwack, G. (1984a) *J. Biol. Chem. 259*, 3173–3180.

Grandics, P., Miller, A., Schmidt, T. J., & Litwack, G. (1984b) *Biochem. Biophys. Res. Commun. 120*, 59–65.

Housley, P. R., & Pratt, W. B. (1983) *J. Biol. Chem. 258*, 4630–4635.

Housley, P. R., & Pratt, W. B. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1572.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.-G., Binart, N., Mester, J., & Baulieu, E.-E. (1984) *Nature (London) 308*, 850–853.

Kovačič-Milivojević, B., LaPointe, M. C., Reker, C. E., & Vedeckis, W. V. (1985) *Biochemistry 24*, 7357–7366.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

LaPointe, M. C., Chang, C.-H., & Vedeckis, W. V. (1986) *Biochemistry 25*, 2094–2101.

Mayrand, S., & Pederson, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2208–2212.

Mendel, D. B., Holbrook, N. J., & Bodwell, J. E. (1985) *J. Biol. Chem. 260*, 8736–8740.

Miller-Diener, A., Schmidt, T. J., & Litwack, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4003–4007.

Puri, R. K., & Toft, D. O. (1984) *Endocrinology (Baltimore) 115*, 2453–2463.

Reichman, M. E., Foster, C. M., Eisen, L. P., Eisen, H. J., Torain, B. F., & Simons, S. S., Jr. (1984) *Biochemistry 23*, 5376–5384.

Reker, C. E., Kovačič-Milivojević, B., Eastman-Reks, S. B., & Vedeckis, W. V. (1985) *Biochemistry 24*, 196–204.

Renoir, J.-M., Buchou, T., Mester, J., Radanyi, C., & Baulieu, E.-E. (1984) *Biochemistry 23*, 6016–6023.

Sanchez, E. R., Toft, D. O., Schlesinger, M. J., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 12398–12401.

Singh, V. B., & Moudgil, V. K. (1985) *J. Biol. Chem. 260*, 3684–3690.

Sullivan, W. P., Vroman, B. T., Bauer, V. J., Puri, R. K., Riehl, R. M., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 4214–4222.

Vedeckis, W. V. (1981) *Biochemistry 20*, 7237–7245.

Vedeckis, W. V. (1983a) *Biochemistry 22*, 1975–1983.

Vedeckis, W. V. (1983b) *Biochemistry 22*, 1983–1989.

Vedeckis, W. V. (1985) in *Hormonally Responsive Tumors* (Hollander, V. P., Ed.) pp 3–61, Academic, New York.

Vedeckis, W. V., Kovačič-Milivojević, B., LaPointe, M. C., & Reker, C. E. (1985) in *Molecular Mechanism of Steroid Hormone Action* (Moudgil, V. K., Ed.) pp 85–110, de Gruyter, Berlin.

Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.

Yamamoto, K. R., & Alberts, B. M. (1976) *Annu. Rev. Biochem. 45*, 721–746.