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EVIDENCE FOR AN ASSOCIATION OF A RIBONUCLEIC ACID WITH THE PURIFIED, UNACTIVATED GLUCOCORTICOID RECEPTOR

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SUMMARY. The unactivated glucocorticoid-receptor complex (GRC) was purified from rat hepatic cytosol (approximately 4000-fold by specific activity) by a procedure developed in our laboratory. Following elution of unactivated GRC from DEAE-cellulose with a 0.05-0.5 M potassium phosphate gradient, a second gradient of 0.5-1.0 M potassium chloride was started. This gradient eluted material at 0.6 M potassium chloride that incorporated [^{32}P] *in vivo* and stained with ethidium bromide. A predominant ethidium bromide stained band of 100-110 nucleotides was observed. The presence of this material was dependent on the presence of highly purified GRC since presaturation of cytosol with 50 μM unlabeled triamcinolone acetonide (TA) precluded the appearance of this material. Experiments with partially purified GRC from CEM-C7 cells incubated *in vivo* with [^{14}C]uridine indicated that the material eluted at 0.6 M potassium chloride incorporated [^{14}C]-uridine. Collectively, these data suggest that a RNA is associated with the purified, unactivated form of the GRC.

Increasing interest has been paid to the possible association of ribonucleic acids with steroid hormone receptors (1-4). The possibility of such an association between a RNA and the glucocorticoid receptor complex (GRC) has been raised by several lines of evidence (5). Unpurified dexamethasone-receptor complexes

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ABBREVIATIONS. Triamcinolone acetonide (TA): [9α-fluoro-11β, 21-dihydroxy-16α,, 17-[(1-methylethylidene)bis(oxy)]pregna-1,4-diene-3, 20-dione]; Mes: 2-(N-morpholino)ethanesulfonic acid; KP: potassium phosphate; KCl: potassium chloride.

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show a binding preference for polynucleotides (1,6), can be inhibited from binding to DNA-cellulose by RNA (1,6), and can be displaced from DNA-cellulose by RNA (1). A major criticism of these studies has been that RNA is primarily acting as a competitor for the DNA-binding site on the receptor. A second major line of evidence has come from studies with RNase. Costello and Sherman (7) showed that RNase increases the amount of GRC sedimenting at the 4-5S (activated) position, while Hutchens et al. (8) reported that the 4-5S form of the receptor could be converted to an 8S form by the addition of a RNase sensitive factor. More recently, RNase has been shown to increase DNA-cellulose binding concommitant with a decrease in sedimentation coefficient (3,9,10). The RNase data are also subject to criticism since it could be argued that digestion of competitive but receptor-unassociated RNA results in the observed increases in DNA-cellulose binding. This is especially possible since these studies made use of unpurified cytosols as the source of GRC. Although these data are suggestive of an association of RNA with GRC, physicochemical data showing this association between purified GRC and RNA are lacking. Here we present direct physical evidence that RNA is associated with purified unactivated GRC.

EXPERIMENTAL PROCEDURES

Materials. [6,7-^{3}H]Triamcinolone acetonide ([^{3}H]TA) (44.0 Ci/mmol) and [^{14}C]uridine (506 mCi/mmol) were purchased from New England Nuclear. Carrier-free [^{32}P]orthophosphate (285 Ci/mg) was from ICN. Bio-Gel A-1.5 m agarose gel, hydroxylapatite (HTP-DNA grade), and electrophoresis reagents were obtained from Bio-Rad, DEAE-cellulose DE-52 was from Whatman, calibration kits for SDS-polyacrylamide gel electrophoresis and Sephadex G-25 PD-10 minicolumns were from Pharmacia, RNase A was from Boehringer Mannheim, NENSORB 20 nucleic acid purification cartridges from DuPont, and Scintiverse II and other reagents from Fisher.

Cell Culture. Human lymphoid CEM-C7 cells were grown in a supplemented Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal and 10% natal calf serum (Hazelton Dutchland, Inc., Denver, PA) as stationary suspension cultures in a humidified atmosphere with 5% CO_2 and 95% air at 37°C. Prior to harvesting cells for cytosol preparation, serum was removed from the media for a 24-48 hour period. Cells were incubated with [^{14}C]uridine for 16-20 hours prior to experimentation.

Animals. Male Sprague-Dawley rats (100-200 g) were adrenalectomized 3-4 days prior to experimentation. For *in vivo* [^{32}P] experiments, rats received 5 mCi [^{32}P] inorganic phosphate intraperitoneally 18 hours prior to experimentation.

Cytosol preparation and receptor purification. Cytosols were prepared and receptors purified as previously described (11,12).

RNA samples were obtained by following the elution of GRC from DEAE-cellulose with a 0.5-1.0 M KCl gradient. RNA peak fractions were combined and either concentrated by acid precipitation for electrophoresis or chromatographed on NENSORB nucleic acid purification cartridges as described by the manufacturer. NENSORB eluted RNA was dried down under a stream of N_2 and assayed for DNA-cellulose binding inhibitory activity. RNA samples were electrophoresed on 7 M urea, 12% polyacrylamide slab gels, followed by ethidium bromide staining.

Assay of GRC. Quantitation of GRC was measured by adsorption on hydroxylapatite as previously described (12). The DNA-cellulose binding assay was used to quantitate activated glucocorticoid-receptor complexes as previously described (12) except that the entire DNA-cellulose pellet was suspended in 5.0 ml scintillation fluid for measuring radioactivity.

RESULTS

Previous data from this laboratory showed that a heavily phosphorylated substance was eluted from the DEAE-cellulose column following purification of rat hepatic GcR complexes when the column was washed with 3 M KCl (13). In the present study, we sought to determine if the heavily phosphorylated material was RNA. Hepatic cytosol was prepared from rats that had received 5 mCi of [^{32}P]orthophosphate and GRC were purified. Following purification of GRC as described (the final purification step being elution of GRC from DEAE-cellulose), the 0.5-1.0 M KCl gradient was started. Aliquots taken for measuring [^{32}P] indicated that the majority of the radioactivity eluted at the 0.6 M KCl position (Fig. 1A). A subsequent gradient of 1.0-2.0 M KCl eluted no further [^{32}P] labeled material from the column. Examination of the [^{3}H]TA-receptor peak fractions and [^{32}P] material on 7 M urea polyacrylamide gels revealed that the 0.6 M KCl sample contained ethidium bromide-staining material. The predominant band migrated to an approximate 100-110 nucleotide position (Fig.2).

To determine if the RNA was specifically associated with the unactivated GRC, cytosol from [^{32}P] injected rats was presaturated with 50 μM unlabeled TA prior to receptor purification.

This concentration of TA prevents purification of GRC and associated subunits by saturating the steroid binding sites of the receptors and thereby preventing their subsequent binding to the deoxycorticosterone derivatized affinity resin. [^{3}H]TA-receptor complexes were absent from the gel-filtration and ion exchange columns and the amount of [^{32}P] labeled material eluted from DEAE-cellulose at 0.6 M KCl was substantially reduced (Fig. 1B). No ethidium bromide staining material was apparent in the [^{3}H]TA-

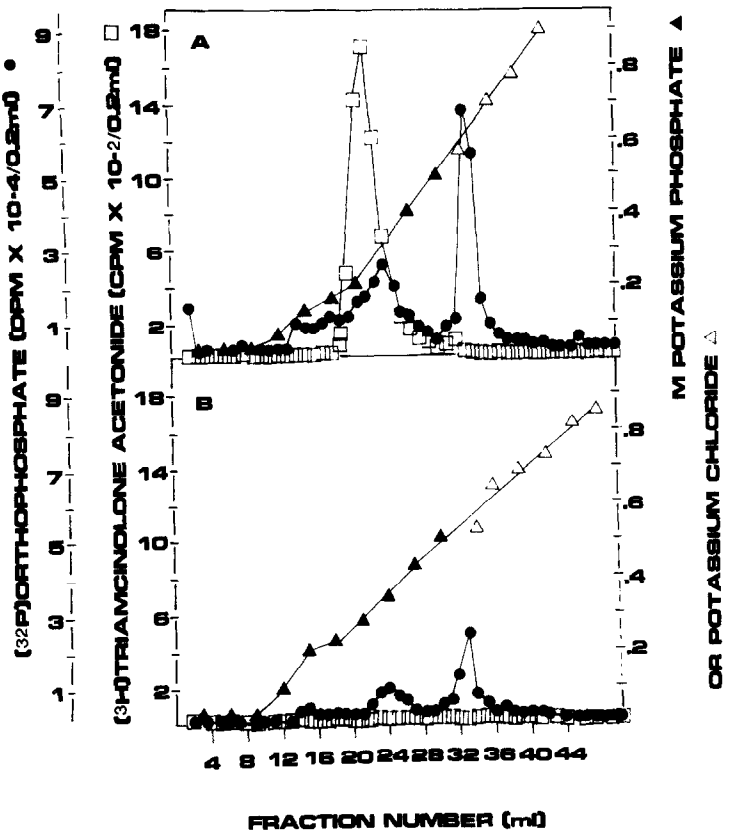

Figure 1. DEAE-cellulose chromatograms of receptor preparations from rats injected with [^{32}P]orthophosphate. Cytosols were either processed for receptor purification (A) or pre-saturated with 50 μM unlabeled TA prior to receptor purification (B). Affinity and gel filtration chromatography preceded DEAE-cellulose chromatography. Linear gradients of 0.05 to 0.5 M KP and 0.5 to 1.0 M KCl were used to elute [^{3}H]TA-receptor complexes and [^{32}P] RNA. One-ml fractions were collected and aliquots taken for measuring radioactivity and salt concentration. Peak [^{3}H]TA and [^{32}P] fractions, as well as fractions at corresponding salt positions from presaturated cytosol, were precipitated for electrophoresis.

receptor sample, or in the DEAE-cellulose fractions at 0.2 M KP or 0.6 M KCl from the unlabeled TA presaturated cytosol (Fig. 2).

To verify that the [^{32}P] labeled material at 0.6 M KCl was in fact RNA, CEM-C7 cells were labeled *in vivo* with [^{14}C]uridine. Cytosol was prepared, cytosolic receptor labeled with [^{3}H]TA *in vitro* and GRC partially purified. Due to receptor losses encountered during purification, the full 3 step purification was not used. Instead, GRC were partially purified using 2 step variations of the 3 step procedure. In the first variation, the affinity chromatography step was omitted and GRC were purified by a combination of gel filtration and ion exchange chromatography.

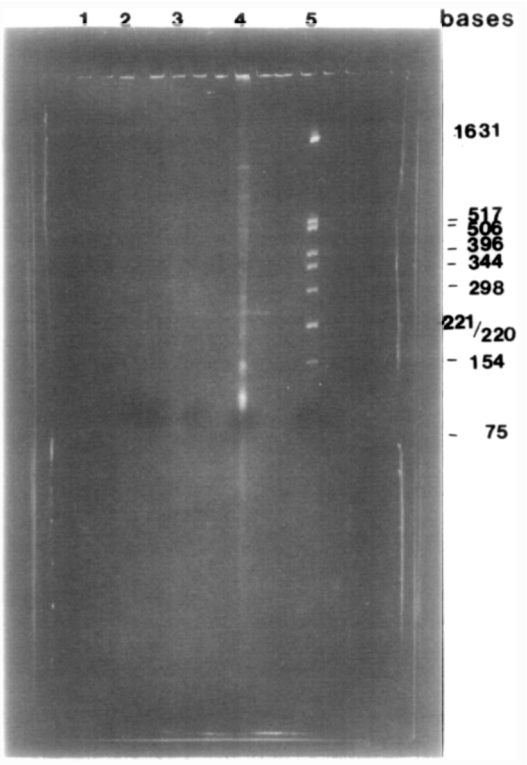

Figure 2. Electrophoretogram of [^{3}H] TA-receptor and [^{32}P] peak fractions from DEAE-cellulose column of presaturated (10^{-7} M unlabeled TA) or non-presaturated cytosols. Precipitated material was electrophoresed on a 7 M urea, 12% polyacrylamide gel. Lane 1: 0.2 M KP fraction from TA-presaturated cytosol; Lane 2: [^{32}P] peak fractions (0.6 M KCl) from TA-presaturated cytosol; Lane 3: [^{3}H] TA peak fractions (0.2 M KP) from non-TA-presaturated cytosol; Lane 4: [^{32}P] peak fractions (0.6 M KCl) from non-TA-presaturated cytosol; Lane 5: Hinf 1 digest of pBR322. The dark circles appearing midway through the gel and at the base of the gel are the xylene cyanol and bromophenol blue dyes, respectively.

In the second variation, the gel filtration step was omitted and GRC were purified by a combination of affinity and ion exchange chromatography. In both cases the [^{3}H] TA-receptor complexes eluted at 0.2 M KP while a peak of [^{14}C] uridine labeled material eluted at 0.6 M KCl (Fig. 3).

DISCUSSION

This study provides the first direct physicochemical evidence that (a) RNA is associated with the highly purified unactivated GRC. At the final purification step of DEAE-cellulose (affinity and gel filtration chromatography preceded ion exchange chromatography) GRC are eluted at 0.2 M KP while the RNA is eluted at

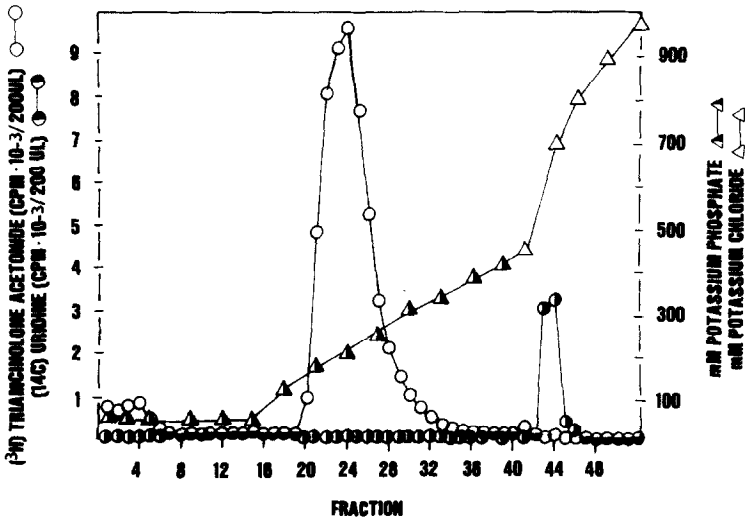

Figure 3. DEAE-cellulose chromatogram of receptor preparation from CEM-C7 cells grown for 18 h in the presence of [^{14}C]uridine. Cytosol was obtained and incubated with [^{3}H]TA 3 h/4°C) and partially purified by gel filtration and ion exchange chromatography. The [^{3}H]TA-receptor peak fractions from the gel filtration column were pooled and applied to the ion exchange column.

0.6 M KCl. That the material eluted at 0.6 M KCl is in fact RNA is indicated by the 1) *in vivo* incorporation of [^{32}P]orthophosphate; 2) ethidium bromide staining and 3) *in vivo* incorporation of [^{14}C]uridine. Previous investigations have provided indirect physicochemical evidence of a GRC-associated RNA. Schmidt et al. (14) have reported that RNase stimulates activation of highly purified GRC while Housley and Pratt (15) reported the removal of a 21,000 dalton component of purified GRC by RNase. More recently, Kovacic-Milivojevic et al. (16) reported the elution of RNase sensitive material at 0.5 M KCl when partially purified GRC from AtT-20 cell cytosol was chromatographed on DEAE-cellulose. This result is consistent with our observation of RNA at 0.6 M KCl when highly purified GRC from rat liver cytosol were chromatographed on DEAE-cellulose.

A specific GRC-RNA association is indicated by several lines of evidence. Most significant is the receptor-dependent presence of RNA at 0.6 M KCl. Secondly, whereas purification of GRC in the activated form could conceivably lead to a non-specific association between receptor and RNA, in the present study GRC were purified in the unactivated form. Moreover, the observation by Economidis and Rousseau (17) argue against the possibility that RNA may associate artifactually with unactivated receptor in the

cytosol. These investigators labeled rat hepatoma cells in culture with [^{14}C]uridine and [^{3}H]dexamethasone mesylate (Dex) and subsequently irradiated these cells to crosslink and thereby stabilize RNA-protein complexes. Cytosol prepared from these cells showed the comigration of the [^{14}C]uridine and [^{3}H]Dex. These results indicate that RNA was bound to the receptor protein in the intact cell. Therefore, it is unlikely that RNA became associated with the receptor in the cytosol during the preparation of cytosol or during the purification of GRC due to an attraction between the polynucleotide binding form of the receptor and RNA. Since 3 different purifications were used to purify GRC from 2 different species and tissue models it is also not likely that the 0.6 M KCl eluted RNA is an artifact of a particular purification step, tissue or species.

The identity of the receptor associated RNA(s) is at present unknown. The elution position from DEAE-cellulose and the 100-110 nucleotide size of the predominant ethidium bromide stained band are suggestive of a tRNA or small nuclear or cytoplasmic RNA (18-19). The reason for the presence of several bands is unclear. One interpretation is that several RNAs are associated with the GRC suggesting a non-specific association. However, this interpretation is inconsistent with the receptor-dependent presence of these bands. A possibility which is more consistent with the data is that nucleolytic degradation may have occurred. If this is the case, then the receptor-associated RNA is larger than 100-110 nucleotides. The biological significance of a GRC-RNA complex is presently under investigation.

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