Association of the glucocorticoid hormone receptor with ribonucleic acid

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The hypothesis that the glucocorticoid hormone receptor interacts with RNA has been tested in cultured rat hepatoma cells. The receptor was covalently labeled with radioactive dexamethasone mesylate, and putative RNA-receptor complexes were stabilized by either cell-free crosslinking using formaldehyde or irradiation of intact cells. After chemical cross-linking in vitro, the receptor displayed the buoyant density of a ribonucleoprotein in CsCl gradients. After photochemical crosslinking in cells labeled with radioactive uridine, the receptor analysed by polyacrylamide gel electrophoresis was carrying labeled ribonucleotides.

Glucocorticoid *Receptor* *Affinity labeling* *Ribonucleoprotein* *Crosslinking*

1. INTRODUCTION

Glucocorticoid hormones control the expression of a small number of transcriptionally active genes by increasing or decreasing mRNA concentration (review [1]). This results from a glucocorticoid receptor-mediated change in the rate of transcription and (or) in mRNA stability. The receptor is an intracellular oligomeric phosphoprotein. Binding of the hormone promotes dissociation and presumably dephosphorylation of the receptor subunits, a process called 'transformation'. The transformed receptor binds to chromatin where it can interact with defined DNA regions, thereby controlling gene expression by unknown mechanisms. Cell-free receptor transformation is stimulated by high ionic strength and by temperature and is blocked by molybdate ions. Transformation can be assessed in vitro by an increased affinity of the receptor for DNA or by a decrease in its sedimentation coefficient.

Abbreviations: dexamethasone, 9α-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione; HTC, hepatoma tissue culture; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine

Earlier studies have suggested that the non-transformed receptor may be associated with RNA. Treatment of the cytosolic receptor with ribonuclease stimulated its transformation [2,3]; this was inhibited by cytosolic RNA [2,4] and by molybdate [3]. In view of this indirect evidence and of the suggestion [1] that this effect of ribonuclease is an artifact, we have approached the question in a more direct way. We show here that the non-transformed receptor of rat hepatoma (HTC) cells, which possess steroid receptors for glucocorticoids only [5], behaves as a ribonucleoprotein after crosslinking in vitro and in vivo.

2. MATERIALS AND METHODS

2.1. *Materials*

Reagent grade materials were used in all experiments. [^{3}H]Dexamethasone (41 Ci/mmol) was from the Radiochemical Centre, Amersham, UK; [^{3}H]dexamethasone 21-mesylate (49 Ci/mmol), and [^{14}C]uridine (50 Ci/mmol) from New England Nuclear, Boston, MA. Dexamethasone and dexamethasone mesylate were gifts from Merck, Sharp and Dohme (Rahway, NJ) and Dr M.V. Govindan, respectively. Ribonuclease A was from

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Boehringer, Mannheim, FRG; it was pretreated at 80°C for 20 min to destroy deoxyribonuclease and protease activity. CsCl was from Bethesda Research Laboratory, Gaithersburg, MD. The M_r markers, phosphorylase *b*, aldolase, serum albumin, ovalbumin and myoglobin were from SERVA, Heidelberg, FRG or from Pharmacia, Uppsala, Sweden. Buffer A contained 20 mM Tricine (Calbiochem, San Diego, CA), 10 mM Na_2MoO_4, and 1 mM EDTA (pH 7.4).

2.2. *Cell culture and labeling procedures*

HTC cells (clone 4) were grown in suspension culture and used in the logarithmic phase of growth, and cytosol was prepared in buffer A [6]. RNA was labeled by incubating the cells in presence of [^{14}C]uridine (3 μCi/ml) for 20 h at 37°C. The receptor was labeled noncovalently by incubating cytosol with 25 nM [^{3}H]dexamethasone for 2–4 h at 4°C. Bound receptor concentration was determined by a charcoal adsorption assay [6]. The receptor was labeled covalently either by adding [^{3}H]dexamethasone mesylate (80 nM) to the cell culture medium 30 min before harvesting the cells [7], or by incubating the cytosol with 50 nM of this steroid for 4 h at 4°C.

2.3. *Gradients*

Sucrose and CsCl gradient centrifugations were performed in a Beckman (Palo Alto, CA) L3-50 ultracentrifuge using a SW50.1 rotor. Linear sucrose (5–20%, w/v) gradients in buffer A were layered with 0.25 ml cytosol and centrifuged at 39000 rpm for 16 h at 4°C. Fractions (0.14 ml) were collected, 10-μl aliquots were made 10% (w/v) with trichloroacetic acid at 4°C and the precipitate collected on glass fiber filters. Radioactivity was measured in a Ready-solv (Beckman) scintillation mixture using a Beckman LS 1800 counter. For CsCl gradients, cytosol or pooled fractions from sucrose gradients were dialysed at 4°C for 4 h against a 10 mM sodium phosphate buffer, pH 7.0 and for 24 h against the same buffer containing 2% (v/v) formaldehyde. The dialysate was mixed with CsCl crystals to give a density of 1.5 g/cm^3. After isopycnic centrifugation (44000 rpm at 22°C for 90 h) 0.16 ml fractions were collected, their density was determined by pycnometry of 10 μl aliquots, and the radioactivity in the acid-precipitable material was measured as described for sucrose gradients.

2.4. *Other methods*

Photochemical RNA-protein crosslinking in intact cells was carried out as described earlier [8]. The cells at a density of 10^7/ml (3 ml per 50-mm Petri dish on ice) were irradiated for 15 min at 3.6×10^5 erg/mm^2 using two Philips TUV15W ultraviolet lamps. Cytosol prepared in buffer A was incubated with ribonuclease A (200 μg/ml) for 30 min at 37°C [9], and concentrated by trichloroacetic acid precipitation [7] for SDS–polyacrylamide gel electrophoresis (PAGE) [10].

3. RESULTS AND DISCUSSION

In a first series of experiments, the cytosol was incubated with the electrophilic ligand dex-

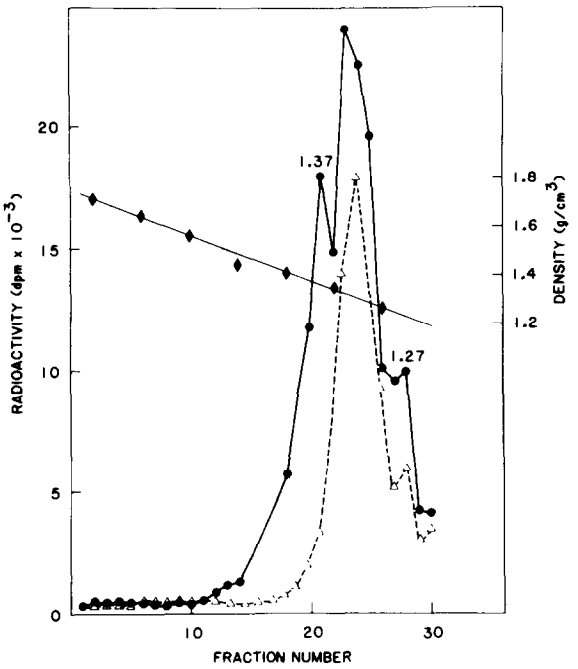

Fig.1. Isopycnic CsCl centrifugation radioactivity profile of cytosol labeled with [^{3}H]dexamethasone mesylate, treated (Δ) or not (●) with ribonuclease, and then crosslinked with formaldehyde. Radioactivity in the fractions was determined by acid precipitation and collection on filters as described in section 2. Lozenges and numbers in the figure refer to density.

amethasone mesylate to label the receptor covalently [11], and treated with formaldehyde so that putative receptor-RNA complexes are stabilized by chemical crosslinking [12–15]. The cytosol was then analysed in CsCl gradients under conditions that allow detection of complexes between proteins and nuclear or cytoplasmic RNA [12–15]. In such gradients, proteins band at 1.25 g/cm^3, hnRNA- and mRNA-protein complexes at 1.40 g/cm^3, ribosomes at 1.50–1.57 g/cm^3, and free RNA sediments at the bottom of the tube (1.9 g/cm^3). Data in fig.1 show that acid-insoluble radioactivity, which presumably includes that associated with the receptor, displayed a heterogeneous profile with peaks at 1.37 and 1.30–1.34 g/cm^3. Treatment of the cytosol with 1 mg/ml ribonuclease A (4°C, 60 min) prior to crosslinking with formaldehyde eliminated the peak at 1.37 g/cm^3. Thus, some of the material labeled with dexamethasone mesylate behaved as a ribonucleoprotein rather than as a free protein.

Since dexamethasone mesylate binds nonspecifically to proteins other than the receptor, the latter was partially purified by centrifuging the covalently labeled cytosol on a sucrose gradient prior to crosslinking. At the low ionic strength used, the nontransformed receptor labelled with [^{3}H]dexamethasone sedimented essentially as a single peak at 8–9 S (fig.2A). When the label was dexamethasone mesylate this peak was still present, with an additional peak at 4 S. A parallel experiment using cytosol incubated with competing nonradioactive steroid showed that the 4 S peak contained mainly proteins that bind the steroid nonspecifically, together with receptor presumably in the transformed conformation (fig.2B). Fractions

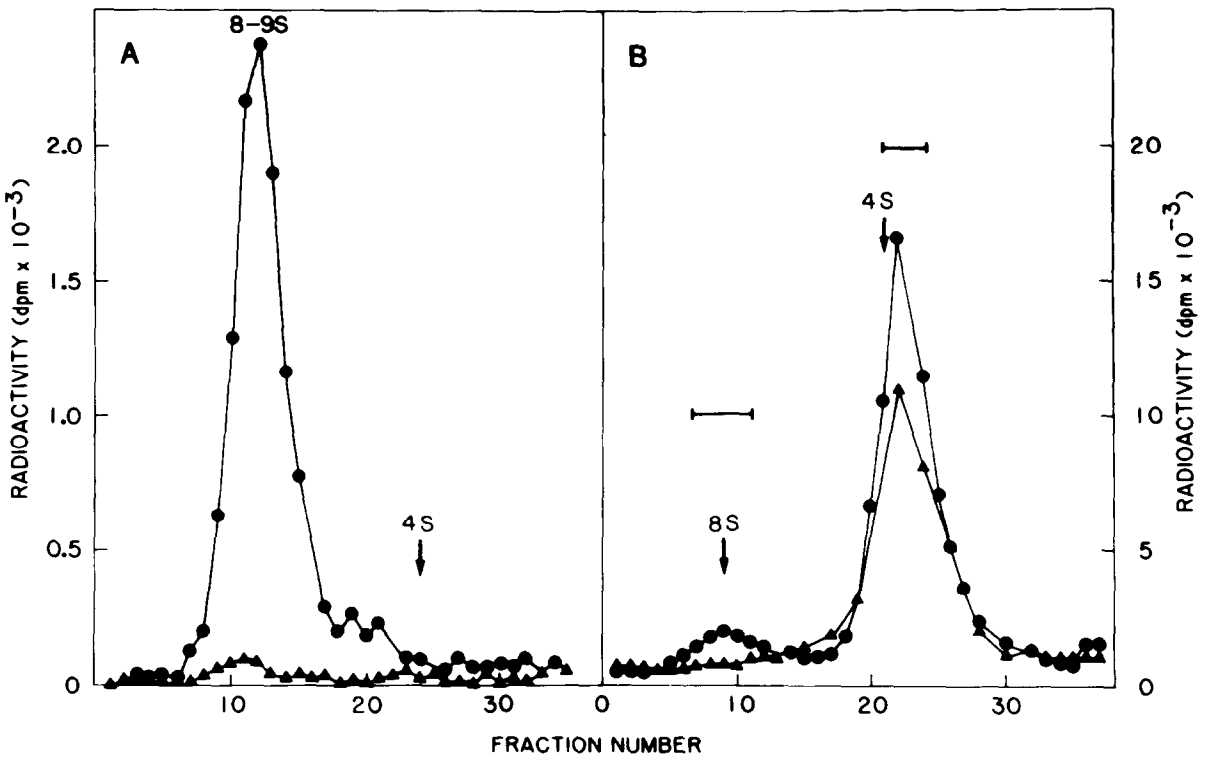

Fig.2. Sedimentation velocity profile, after centrifugation on 5–20% sucrose gradients, of cytosol labeled with [^{3}H]dexamethasone (A) or [^{3}H]dexamethasone mesylate (B) in the absence (●) or presence (▲) of 200-fold excess competing nonradioactive steroid. Prior to counting the fractions, free steroid was eliminated either by charcoal adsorption (A) or by acid-precipitation on filters (B). Note the difference in scale between panels A and B. M_r markers were aldolase, serum albumin, and myoglobin. Fractions under the horizontal bars in B were pooled for the experiment shown in fig.3.

from the 4 S and 8–9 S regions of the gradient containing material labeled with [^{3}H]dexamethasone mesylate were pooled, crosslinked with formaldehyde, and equilibrated in CsCl gradients. The radioactivity profiles (fig.3) show that the material under the 4 S peak of the sucrose gradient equilibrated with the free proteins at 1.28 g/cm^3, while the nontransformed receptor banded at 1.37 g/cm^3, and with a better resolution than when using unpurified cytosol (see fig.1). The ribonucleoprotein nature of the material banding at 1.37 g/cm^3 was further supported by the fact that prior ribonuclease treatment shifted its density to 1.28 g/cm^3 (not shown). It is also supported by the data shown in fig.4. In this experiment, stable RNA was labeled by growing the cells in the presence of [^{14}C]uridine, following which the cytosol prepared from these cells was incubated with [^{3}H]dexamethasone mesylate to label the receptor. Sucrose gradient analysis of the cytosol showed the expected two peaks of macromolecule-bound steroid and a broad profile of uridine-labeled material. CsCl analysis of the crosslinked pooled fractions under the 8–9 S peak again showed a band of macromolecule-bound dexamethasone mesylate at 1.37 g/cm^3, now coinciding with a minor RNA peak. The rest of the RNA either formed other complexes (1.40 g/cm^3) or sedimented at the bottom of the tube.

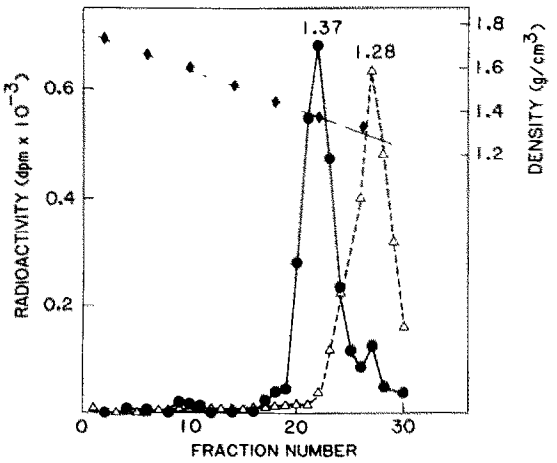

Fig.3. Isopycnic CsCl centrifugation radioactivity profile of pooled fractions under the 8 S (●) or the 4 S (△) peaks of the gradient shown in fig.2B. The pooled fractions were dialysed, cross-linked, and prepared for centrifugation as described in section 2. For other details, see fig.1.

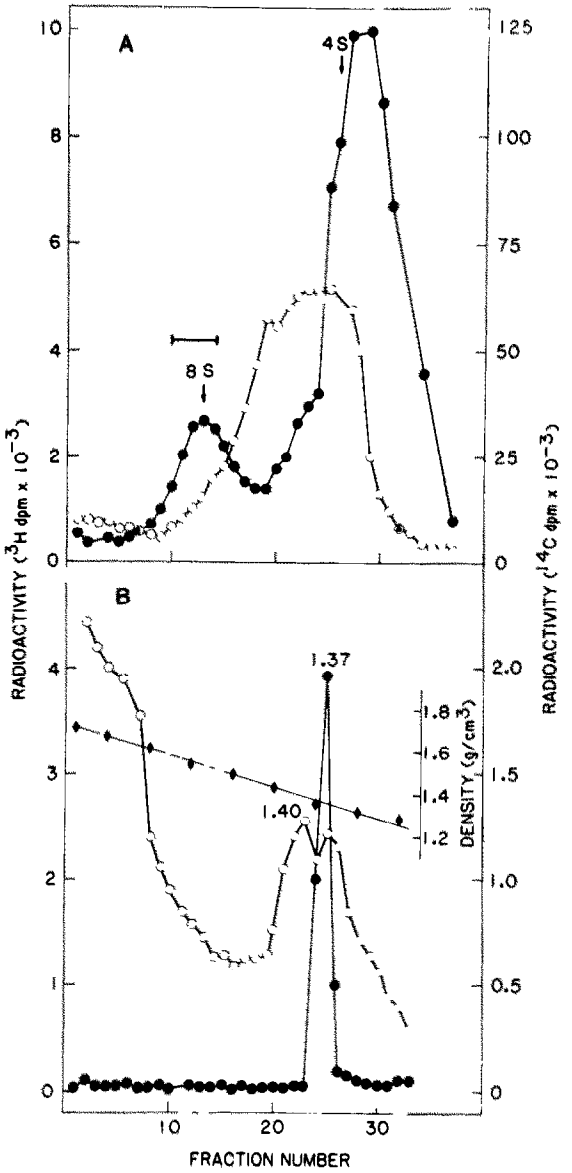

Fig.4. (A) Sedimentation velocity profile, after centrifugation on 5–20% sucrose gradients, of double-labeled cytosol. RNA was labeled in vivo by incubating the cells with [^{14}C]uridine (○) and the receptor was labeled in vitro by incubating the cytosol with [^{3}H]dexamethasone mesylate (●). For other details, see fig.2B. (B) Isopycnic CsCl centrifugation profile of the pooled sucrose gradient fractions corresponding to the 8 S receptor peak in panel A. Experimental conditions were identical to those in fig.3.

Because RNA may associate artifactually with proteins in vitro [16,17], an experiment was designed to try and detect receptor-RNA complexes that could occur in vivo. When intact cells are irradiated at 254 nm, protein and RNA molecules in direct contact can become photochemically crosslinked [8]. RNA and the receptor were now labelled in intact cells by incubating them with [^{14}C]uridine and [^{3}H]dexamethasone mesylate and the cells were irradiated as described in section 2. Cytosol was prepared, treated with ribonuclease, and the proteins analysed by SDS–PAGE. In such gels, the receptor migrates with an apparent M_r around 90000 [7]. Any protein that was in contact with the labeled RNA at the time of irradiation is expected to carry [^{14}C]uridine-labeled ribonucleotides left over after RNA digestion [18]. The electrophoretic profile (fig.5) shows that one of these proteins migrated where the receptor is expected to do so, and that it was labeled with the steroid ligand.

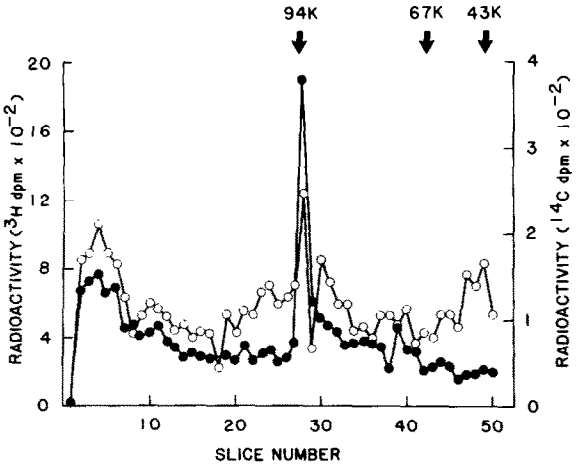

Fig.5. SDS–PAGE of proteins crosslinked to nucleic acids in vivo. The receptor and RNA were both labeled in intact cells as described in section 2 using [^{3}H]dexamethasone mesylate (●) and [^{14}C]uridine (○), respectively. The cells were irradiated as described, the cytosol was treated with ribonuclease, concentrated by precipitation with trichloroacetic acid, resuspended in sample buffer, and analysed on a 10% polyacrylamide slab gel. After fixation with acetic acid and methanol, the gel was cut into 1.2-mm slices which were dissolved in H_2O_2 and counted for radioactivity. A superimposable profile was found in a parallel experiment using [^{3}H]uridine to increase the labeling of RNA. M_r markers were phosphorylase *b*, serum albumin, and ovalbumin (arrows).

Taken together, these results provide direct evidence for a possible association between the glucocorticoid receptor found in the cytosol of HTC cells and an RNA. The physiological significance of this finding is not known. Such an RNA could maintain the receptor in the non-transformed state by 'covering' the DNA-binding, or another, site on the receptor. This must be reconciled with the fact that nuclear glucocorticoid-receptor complexes are also sensitive to ribonuclease treatment [19,20]. However, nuclear-bound steroid receptors might occur in the non-transformed state [21]. A receptor-associated RNA also could allow receptor translocation across the nuclear membrane, in the same way as the RNA-containing 'signal recognition particle' participates to injection of secretory proteins into the endoplasmic reticulum [22]. Another possibility is that DNA recognition by the receptor involves a receptor-associated RNA molecule. Alternatively, such an RNA might play a role in the post-transcriptional effects of the receptor. Clarification of these speculative but exciting issues (small RNAs can behave as biochemical catalysts, see [23]) requires characterization of this RNA, a work in progress in our laboratory.

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