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MAJORITY OF RNA WHICH CO-PURIFIES WITH UNACTIVATED RAT HEPATIC GLUCOCORTICOID–RECEPTOR COMPLEXES IS NONSPECIFIC AND NOT RECEPTOR-ASSOCIATED

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Summary—Molybdate-stabilized, unactivated rat hepatic glucocorticoid–receptor complexes were purified by a three-step procedure which includes affinity chromatography, gel filtration and anion exchange chromatography. Following elution of unactivated steroid–receptor complexes from the final DEAE-cellulose column, RNA which remained bound to the anion exchange resin was eluted with 1 M KCl. This RNA was small and heterogeneous in size. Equivalent amounts of RNA were detected after a mock purification which was devoid of receptors, suggesting that the presence of this RNA is not dependent on that of receptors. Both a [^{32}P]DNA complementary to the RNA eluted from DEAE-cellulose and a [^{32}P]DNA probe synthesized from total rat liver RNA gave similar results when hybridized to total rat liver RNA. These data indicated that the RNA which co-purified with unactivated receptors through the first two steps was very similar to total RNA in overall composition. Virtually identical hybridization patterns were also detected when end-labeled probes generated from the DEAE-cellulose eluted RNA or total liver RNA were hybridized to total genomic rat DNA, suggesting that the RNA eluted from the anion exchange resin is not specific or unique.

Although these results do not exclude the possibility that there could be specific RNA species associated with the unactivated glucocorticoid receptor, they do indicate that the majority of the RNA eluted from DEAE-cellulose following elution of receptor complexes appears indistinguishable from total rat liver RNA and can be detected in parallel mock purifications.

INTRODUCTION

The ability of glucocorticoid hormones to enhance the rate of transcription of specific genes is mediated by an intracellular receptor protein which binds these steroids with high affinity and specificity. Initially the glucocorticoid–receptor complex resides in the cytoplasm in a form, termed "unactivated" or "untransformed", which is incapable of binding to DNA. These unactivated complexes can be purified from rat hepatic tissue in the presence of molybdate as large heterooligomeric complexes with an apparent molecular weight slightly in excess of 300 kDa, and a sedimentation coefficient of 9–10S [1]. After removal of molybdate, these cytoplasmic glucocorticoid–receptor complexes, in either crude [2] or purified [1, 3] preparations, can be thermally converted to a smaller form, termed "activated" or "transformed". This activated form, which is capable of binding to DNA, has an apparent molecular weight of approximately 94 kDa [4], and a sedimentation coefficient of 4S [5]. Although the activated form of the glucocorticoid receptor is a single protein [6] which contains both steroid and DNA binding domains, the additional components or subunits that triple the apparent molecular weight of the receptor in its unactivated form have not been completely elucidated. It has been speculated that this larger form of the receptor may consist of one molecule of the 94 kDa steroid-binding protein itself, a low molecular weight activation inhibitor [7, 8], a dimer of the 90 kDa heat shock protein [9], and possibly RNA [10–12]. Likewise it has been suggested that conversion of these unactivated glucocorticoid–receptor complexes to the DNA-binding form is accompanied by dissociation of some, if not all, of these components from the receptor monomer [9, 13–15].

Experiments conducted in numerous laboratories have indicated that RNA may be associated with the activated form of the glucocorticoid receptor (reviewed in Ref. [11]). The binding of activated glucocorticoid, as well as other steroid–receptor complexes to DNA can be inhibited or displaced by RNA [16, 17], while RNase treatment has been reported to enhance receptor binding to DNA [14, 18, 19]. In several cases this inhibitory activity of RNA appears to be dependent on the base sequence of the RNA, suggesting some specificity [20, 21]. Vedeckis and his

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colleagues[22, 23] have reported that low molecular weight transfer RNA may bind to the activated glucocorticoid receptor and by so doing may play a physiological role with regard to receptor regulation of gene expression. Data generated with partially proteolyzed glucocorticoid–receptor complexes have suggested that RNA may not simply compete for the DNA binding site of the activated protein but may actually interact with a separate site which is specific for RNA and which may allosterically regulate DNA binding [24].

The possibility that RNA may also be associated with the unactivated glucorticoid–receptor complex is more controversial and is the subject of this report. Several years ago Kovacic-Milliojevic *et al.* [22] reported that the unactivated 9–10S receptor from AtT-20 rat pituitary cells is free of RNA and that the receptor becomes associated with RNA only after its conversion to the 4S activated form. In contrast, Economidis and Rousseau[12] found that the unactivated receptor of rat hepatoma (HTC) cells behaves as a ribonucleoprotein after crosslinking either *in vitro* with formaldehyde or *in vivo* by irradiation of intact cells. More recently, a small RNA species has been found to be associated with unactivated rat heptatic glucocorticoid–receptor complexes immunoabsorbed to protein-A-Sepharose via a specific receptor monoclonal antibody [10]. We have also previously addressed this possible association with RNA by using unactivated receptor complexes purified to near homogeneity by a three-step scheme which includes affinity chromatography, gel filtration and anion exchange chromatography [1]. Our preliminary data [25] demonstrated that after elution of the unactivated glucocorticoid–receptor complexes from DEAE-cellulose, which is the last step in the three-step purification scheme, a second higher salt eluate was recovered which appeared to contain RNA.

In the series of experiments reported here we have extended and expanded this protocol to include hybridization of the DEAE-cellulose-eluted RNA with total rat liver RNA as well as genomic DNA. Taken collectively our data suggest that RNA does co-purify with the receptor through the first two selective fractionation steps, but the majority of this RNA is nonspecific with respect to sequence and is not receptor-associated.

MATERIALS AND METHODS

Preparation of hepatic cytosol

Adrenalectomized male Sprague–Dawley rats (175–200 g) were purchased from Hormone Assay Laboratories Inc. The rats were fed a normal chow diet, maintained on 0.9% NaCl, and used 5–8 days after surgery. Following anesthetization by intraperitoneal injection with Ketamine, rat livers were purfused *in situ* via the portal vein with 50 ml cold 0.9% NaCl and then with 50 ml buffer A (50 mM potassium phosphate, 10 mM sodium molybdate, 2 mM DTT, and 10 mM thioglycerol, pH 7.0 at 4°C). The excised and minced livers were weighed and then homogenized in 1 vol of ice-cold buffer A with a Brinkman Polytron (PT-10-35) homogenizer. After the crude homogenate was centrifuged at 4000 ***g*** for 15 min at 4°C, the supernatant was centrifuged at 105,000 ***g*** for 1 h at 0–4°C. The resulting cytosolic fraction was stored under liquid nitrogen for subsequent receptor purifications.

Purification of unactivated glucocorticoid receptors and recovery of RNA from DEAE-cellulose

Starting with approximately 70 ml of hepatic cytosol, glucocorticoid–receptor complexes stabilized in their unactivated form by the presence of 10 mM Na_2MoO_4 (Sigma) were purified to near homogeneity using minor modifications [14] of a published scheme [1] which began with adsorption of unbound receptors to a deoxycorticosterone-derivatized agarose and elution with [1,2,4(*N*) ^{3}H]triamcinolone acetonide ([^{3}H]TA; 28 Ci/mmol; Amersham). This was followed by gel filtration on Bio-Gel A-1.5 m agarose (Bio-Rad), and DEAE-cellulose (Whatman) chromatography. Previously published data [1, 33] indicated that when the DEAE-cellulose eluted receptors are analyzed by SDS–polyacrylamide electrophoresis a single protein band of approximately 94 kDa is detected with Coomassie blue, while subsequent staining with silver reveals several additional minor bands of lower molecular weight. In the present studies, except for the fact that rat liver cytosol was first incubated for 16 h with 2 μM nonradioactive triamcinolone acetonide, mock purifications were performed in an identical manner. Following elution of the unactivated receptor complexes from DEAE-cellulose with either a 50–500 mM potassium phosphate pH 7.0 (KP) gradient or a 150–500 mM KCl gradient in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.0, the DEAE-cellulose bound RNA was eluted in approximately 1 ml fractions with 10 ml 1 M KCl, 10 mM MES pH 7.0. The first five fractions were pooled and nucleic acids were precipitated by adjustment to a final concentration of 0.2 M sodium acetate pH 5.0 and addition of two volumes of ethanol. After incubation overnight at −20°C the precipitate was collected by centrifugation for 15 min at 10,000 ***g***. The material was dissolved in 0.6 ml H_2O, reprecipitated as above, and collected by centrifugation for 15 min at 13,600 ***g*** in a microcentrifuge. Ethanol (70%) was added, the material centrifuged again as before, and the final pellet dissolved in 50 μl H_2O.

Synthesis of radioactive probes

The RNA recovered from DEAE-cellulose was directly 5′-end-labeled by incubating one-third of the recovered material in a 24 μl reaction containing 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mM spermidine, 0.1 mM EDTA, 25 μCi [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear), and 2.5 units T_4 polynucleotide

kinase (New England Biolabs). After 30 min at 37°C the reaction was stopped by the addition of EDTA pH 8.0 to a final concentration of 20 mM and 100 μg yeast RNA. Following extraction with chloroform:isoamyl alcohol (24:1), the aqueous phase was adjusted to 2 M ammonium acetate, then two volumes of ethanol were added. After 15 min on dry ice and 10 min centrifugation at 13,600 *g*, the pellet was dissolved in 10 mM Tris–HCl pH 8.0, 0.1 mM EDTA. For the experiments shown in Fig. 4, RNA from both the normal and the mock purification incorporated about 2–2.5 × 10^6 cpm under these conditions. For labeling total rat liver RNA, which was isolated as described below, essentially the same kinase reaction conditions were employed. Synthesis of a complementary DNA copy of the RNA was accomplished in a 20 μl reaction mixture containing one-third of the recovered RNA, 50 mM Tris–HCl pH 8.7, 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 250 μM each dCTP, dGTP, and dTTP, 1.5 μM dATP (including labeled dATP), 60 μCi [α-^{32}P]dATP (3000 Ci/mmol, New England Nuclear), 10 μg/ml hexameric oligodeoxynucleotide mixture (Pharmacia), and 6 units AMV reverse transcriptase (Boehringer Mannheim Biochemicals). The reaction was incubated at 44°C for 30 min and was then terminated with EDTA, extracted, and ethanol precipitated exactly as above for the terminally-labeled RNA. Under these conditions incorporation of the radioactive precursor averaged approximately 1 × 10^6 cpm for RNA from both receptor and mock preparations. The conditions for synthesizing cDNA from total rat liver RNA were as follows: 50 mM Tris–HCl pH 8.0, 40 mM KCl, 8 mM $MgCl_2$, 7 mM β-mercaptoethanol, 50 μM each dATP, dGTP, and dTTP, 25 μCi [α-^{32}P]dCTP (3000 Ci/mmol, New England Nuclear), 500 μg/ml deoxynucleotide hexamers as above, 18 units AMV reverse transcriptase, and 8 μg total rat liver RNA in a 20 μl reaction volume. The reaction mixture was incubated at 37°C for 45 min and incorporated radioactivity was about 2.5 × 10^6 cpm per μg RNA.

Isolation of total RNA and genomic DNA from rat liver

Total cellular RNA was purified from the liver of an adrenalectomized rat using initial dissolution in guanidine–HCl essentially as described previously [26], except the precipitation of the RNA in 3 M sodium acetate was omitted. Prior perfusion of the liver was carried out as already described. Rat genomic DNA, which was a generous gift from Dr Richard Maurer, was purified from the liver essentially as described previously [27].

Gel electrophoresis and molecular hybridization

Total rat liver RNA was size-fractionated by gel electrophoresis in a 2% agarose, formaldehyde-containing gel as described previously [28]. The sample loading buffer was as described [28] except that instead of using agarose slurry, the sample was adjusted to 10% glycerol, 0.02% SDS, 0.2% Bromophenol blue and then heated for 5 min at 60°C before loading. Transfer of the RNA from the gel to a Genescreen membrane was done as before [28]. After transfer the membrane was submerged in 25 mM sodium phosphate pH 6.5, and the surface was rubbed to remove residual agarose. Cross-linking of the RNA to the membrane was accomplished by exposure to 254 nm u.v. light (580 μW/cm^2) at a distance of 15 cm for 3 min, as recommended by the manufacturer (New England Nuclear). The membrane was then placed in a vacuum oven for 2 h at 80°C. RNA molecular weight markers, from Bethesda Research Labs, were stained on the membrane with methylene blue as outlined [29] with the exception that all times were reduced to 5 min. RNA obtained in the DEAE-cellulose eluate following either normal or mock receptor purification was itself subjected to gel electrophoresis and transfer to Genescreen exactly as described here, except that the amount of agarose in the gel was 2.5% (dissolved by autoclaving for 10 min.).

For hybridization with radioactive probes, filter strips with total RNA were first prehybridized for 5 h at 60°C in 7 ml 0.2X SSC (1X SSC = 0.15 M NaCl, 0.015 M Na-citrate), 50 mM sodium phosphate pH 6.5, 0.5% SDS, 25 μg/ml *E. coli* DNA (sheared by depurination [30]), 0.2% crystalline bovine serum albumin (BSA; Behring), 0.2% polyvinylpyrrolidone, 0.2% Ficoll [31]. Hybridizations were carried out for 18 h at 60°C in a 5 ml solution with the same composition plus radioactively labeled cDNA (0.2–0.5 × 10^6 cpm). Prior to addition to the hybridization mix, the probe, in one-tenth the final volume, was denatured together with 125 μg *E. coli* DNA by boiling for 5 min in 0.3 N NaOH, followed by quick cooling on ice and addition of HCl to 0.3 N. After hybridization, the membranes were washed twice for 5 min each at room temperature in 2X SSC, 0.1% SDS and twice for 60 min each at 60°C in 0.2X SSC, 0.1% SDS.

Rat genomic DNA was digested with the restriction enzyme HindIII (New England Biolabs) and was resolved by electrophoresis for 15 h at 25 V in an 0.8% agarose gel in Tris–acetate buffer [32]. DNA molecular weight markers (Bethesda Research Labs), were stained in the gel with 1 μg/ml ethidium bromide. The gel was soaked in 0.25 N HCl for 10 min to depurinate the DNA and was then placed in 0.4 N NaOH for 5 min to denature the DNA. Transfer of the DNA to a Zetaprobe (Bio-Rad) membrane was done by blotting in 0.4 N NaOH. The membrane was rubbed in 2X SSC and baked as described above for Genescreen.

Filter strips with DNA were pre-hybridized for 5 h at 37°C in 7 ml of 50% formamide (EM Corp, adsorbed for 30 min with Bio-Rad AG501–X8[D]), 5X SSC, 10 mM Hepes–NaOH pH 7.5, 1 mM EDTA pH 8.0, 1% SDS, 50 μg/ml *E. coli* DNA (sheared as above), 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll. Hybridizations were carried out for 14 h at

37°C in 5 ml of 50% formamide, 5X SSC, 10% dextran sulfate (Pharmacia), 10 mM Hepes-NaOH pH 7.5, 1% SDS, 50 μg/ml *E. coli* DNA, 0.06% BSA, 0.06% polyvinylpyrrolidine, 0.06% Ficoll, and 1×10^6 cpm of 5′-end-labeled RNA that had been boiled for 5 min, then placed on ice, prior to addition to the hybridization mixture. After hybridization, filters were washed twice for 5 min each at room temperature in 2X SSC, 0.1% SDS and twice for 45 min each at 60°C in 0.5X SSC, 0.1% SDS.

For all prehybridizations and hybridizations, filters were placed in Seal-A-Meal bags and kept in an incubator without agitation. After washing, membranes were wrapped in Saran Wrap, and exposed to XAR5 film (Kodak) at −70°C with a Lightning Plus Intensifying Screen (Dupont).

RESULTS

Purification of the unactivated glucocorticoid receptor

The molybdate-stabilized unactivated glucocorticoid-receptor complexes were purified from rat hepatic cytosol by a three-step procedure which included affinity chromatography, gel filtration and anion exchange chromatography. As seen in Fig. 1, the purified [^{3}H]triamcinolone acetonide (TA)-receptor complexes were eluted from DEAE-cellulose as a single peak of bound radioactivity. The data presented in Fig. 1 also demonstrate that in a mock purification, in which the rat liver cytosol was preincubated with 2 μM nonradioactive TA to block subsequent binding of receptors to the affinity resin, a similar peak of receptor bound [^{3}H]TA was not detected. Western blot analysis with the BUGR-2 anti-receptor monoclonal antibody [33] also revealed dramatically reduced levels of immunoreactive material in the mock purification when compared with the normal purification (data not shown).

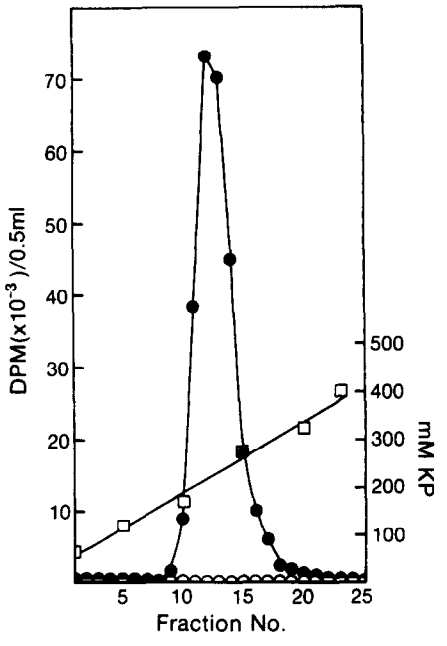

Fig. 1. DEAE-cellulose chromatograms depicting final step in normal (●) and mock (○) purification of unactivated rat hepatic glucocorticoid-receptor complexes. Linear potassium phosphate gradient (□).

Demonstration of the presence of RNA in purified receptor preparations

In order to determine whether RNA was present in the preparations of purified unactivated receptor, the following experiment was performed. The receptor was purified through the first affinity chromatography step, which exploits the specific steroid-binding properties of the protein. It was then subjected to size fractionation on Bio-Gel and the very large 300 kDa complexes were recovered. In the final step, the pooled Bio-Gel fractions containing these complexes were applied to a DEAE-cellulose column. After elution of the unactivated [^{3}H]TA–receptor complexes from DEAE-cellulose, the anion exchange resin was further eluted with 1 M KCl. Any material detected in this second eluate necessarily must have co-purified with the receptor complexes through the first two purification steps. By reading the absorbance of the eluate at 260 nm (A_{260}), it was determined that nucleic acid moieties could be present. However, the values were too low to be an accurate measure of the quantity, which generally appeared to be less than 1 μg.

In order to further analyze the eluate for the presence of RNA, we radioactively labeled the material in the eluate by the addition of [^{32}P]phosphate to free 5′ termini and then size fractionated the RNA on a denaturing agarose gel. As shown in Fig. 2, lane A, the RNA recovered in the 1 M KCl eluate of the DEAE column was small and heterogeneous in size, migrating as a broad distribution of species less than 250 bases in length. Although this type of agarose gel does not provide high resolution in the size range observed, it does demonstrate unequivocally that the RNA was of relatively small size, with essentially none of it being greater than about 250 nucleotides in length.

In a mock purification which lacked detectable receptors (Fig. 1), RNA was also present in approximately the same quantity, as judged by A_{260} and incorporation of radioactivity. This material, shown in Fig. 2, lane B, had an identical migration pattern as observed for the RNA from a normal receptor purification. These results suggested that although RNA was found in purified receptor preparations, its presence was not dependent on that of receptors.

Synthesis of a complementary probe to the recovered RNA and hybridization to total liver RNA

Because the receptor purification scheme was developed for maximal recovery of the receptor protein, the conditions were not necessarily optimal for maintaining RNA intact. Thus it is possible that the RNA recovered in the receptor and mock preparations had been degraded and that the size observed by gel

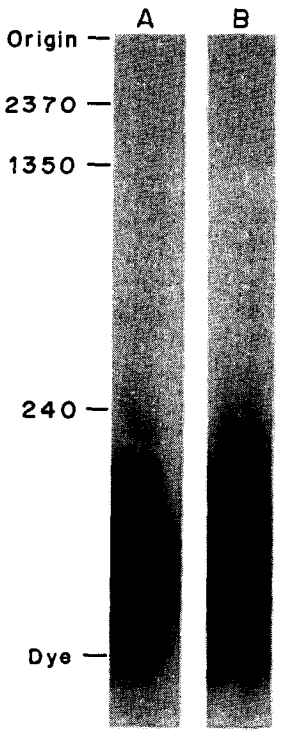

Fig. 2. RNA recovered from DEAE-cellulose from either a normal (lane A) or a mock (lane B) receptor preparation. Following elution from the anion exchange column, the RNA was 5′-end labeled, resolved by electrophoresis in a 2.5% agarose gel containing formaldehyde, transferred to Genescreen, and detected by exposure of XAR5 film as described in Materials and Methods. The sizes of RNA molecular weight markers, in number of bases, are indicated on the left.

electrophoresis of the final material, as shown in Fig. 2, did not accurately reflect its original size.

In order to overcome this problem, we synthesized a radioactive complementary DNA copy of the RNA recovered from the DEAE column, for both normal receptor and mock preparations. This cDNA should represent all sequences present because its synthesis was initiated from random primers. This probe was then hybridized to total rat liver RNA which had been size fractionated by gel electrophoresis and transferred to a filter. The procedure used for isolation of total liver RNA was designed to yield intact RNA. Thus even though the RNA co-purifying with the receptor may itself have been degraded, its cDNA copy should hybridize to RNA in the total liver preparation that was still of the original size.

The data from this analysis are shown in Fig. 3. The cDNA probes from RNA of either the normal (lane A) or mock (lane B) receptor preparations gave essentially the same results. They hybridized to a very broad range of higher molecular weight RNA, clearly different from the small size of the RNA found in Fig. 2. Some of the hybridization appeared in the region of ribosomal RNA, labeled 28S and 18S. There was also hybridization to RNA smaller than ribosomal RNA, which probably was not due to substantial degradation of the total liver RNA, since hybridization with an albumin cDNA probe demonstrated that the RNA on the filter was primarily intact (data not shown). As a control we also prepared ^{32}P-labeled cDNA from total liver RNA and hybridized it to total RNA on a filter (Fig. 3, lane C). This filter was identical to those used to hybridize with the probes made from normal and mock receptor preparations described above, and the results were similar, although there was somewhat more hybridization in the 28S region. All of the filters were intentionally overloaded with regard to ribosomal RNA, in order to detect any minor species of RNA that may have hybridized to the probes.

This experiment showed that the probe made from the RNA eluted from DEAE-cellulose gave a very similar hybridization pattern as the probe synthesized from total rat liver RNA. Thus the RNA that co-purified with the receptor through the first two steps was very similar to total rat liver RNA in overall composition. Furthermore, the fact that indistinguishable RNA was also recovered from mock receptor preparations suggested that most, if not all, of this RNA was present irrespective of the presence of the receptor. Whether there was also a small subset

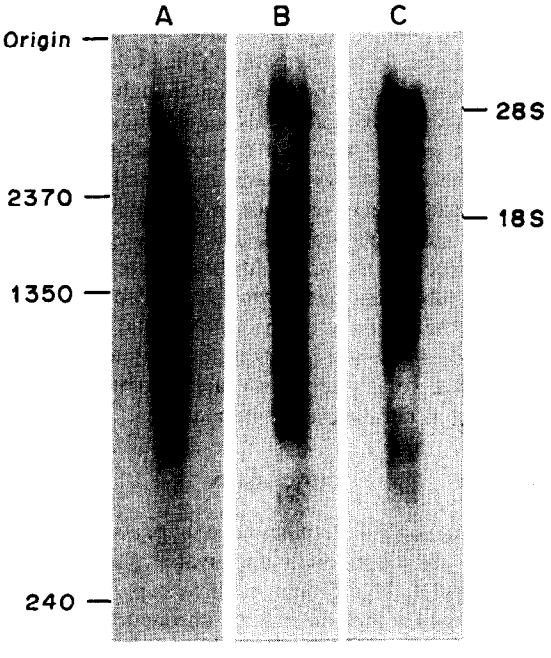

Fig. 3. ^{32}P-labeled cDNA, synthesized from RNA recovered from DEAE-cellulose, hybridized to total rat liver RNA. RNA was eluted from DEAE-cellulose in either a normal (lane A) or a mock (lane B) receptor preparation. The RNA was used as a template to synthesize a radioactive cDNA copy and these probes were hybridized to total rat liver RNA, which had been resolved by electrophoresis and bound to Genescreen. As a control, [^{32}P]cDNA was made from total rat liver RNA and hybridized to exactly the same RNA on the Genescreen filter (lane C). The sizes of RNA molecular weight markers, in number of bases, are indicated on the left and the position of migration of 28S and 18S ribosomal RNA is shown on the right.

of RNA molecules specifically associated with the glucocorticoid receptor cannot be determined from this experiment because of these other nonspecific species.

Hybridization of the recovered RNA to rat genomic DNA

Another approach to analyzing the composition of the RNA that co-purified with the glucocorticoid receptor was to hybridize it to total genomic rat DNA in order to determine whether unique hybridization products could be detected. For the experiments described here, rat genomic DNA was digested with the restriction enzyme HindIII, size-fractionated by gel electrophoresis, and transferred to a filter. The filters were then hybridized with radioactive probes, which were the terminally-labelled RNAs themselves as used for the experiment described in Fig. 2. Essentially identical results (data not shown) were obtained with random-primed cDNA, as used in Fig. 3. The RNA obtained from a normal receptor purification hybridized to several distinct bands at 9.1, 6.1, 5.5, 3.9, 2.2, and 1.8 kb, as shown in Fig. 4, lane A. As a control, an end-labeled probe generated from total rat liver RNA was also hybridized to filter-bound DNA and a virtually identical pattern of hybridization was obtained (Fig. 4, lane C). RNA present in a mock preparation also hybridized to the rat DNA with a similar pattern (Fig. 4, lane B.). All of the same bands hybridized even though the relative intensity of individual bands varied somewhat with this last probe. Although the explanation for this difference is not known, it was reproducible with the same pattern being observed in four independently-isolated mock preparations. Thus, although our data do not exclude the possibility that there could be a specific RNA associated with the glucocorticoid receptor, the majority of the RNA found in purified unactivated receptor preparations was indistinguishable from total liver RNA and furthermore was detected in parallel mock preparations.

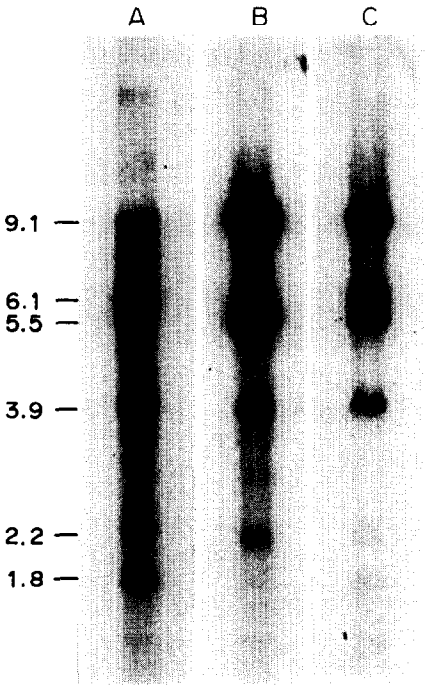

Fig. 4. Terminally-labeled RNA, recovered from DEAE-cellulose, hybridized to total rat genomic DNA. RNA was eluted from DEAE-cellulose in either a normal (lane A) or a mock (lane B) receptor preparation. The RNA was radioactively labeled at the 5′-end and hybridized to rat genomic DNA that had been digested with HindIII, resolved by gel electrophoresis and transferred to a Zetaprobe membrane. In lane C, total rat liver RNA was 5′-end-labeled and hybridized to rat genomic DNA. The approximate size of each of the major bands, determined by comparison to DNA molecular weight markers, is shown on the left in kilobases.

DISCUSSION

Several published studies have suggested that RNA may be associated with unactivated glucocorticoid receptors [10, 12]. Using a three-step purification scheme which includes affinity chromatography, gel filtration and anion exchange chromatography, Grandics *et al.* [34] reported that both unactivated hepatic glucocorticoid receptors as well as an unidentified 24 kDa macromolecule incorporated radioactive phosphate after an *in vivo* injection of [^{32}P]orthophosphate into adrenalectomized rats. It was speculated that the phosphorylated 24 kDa species, which co-eluted from DEAE-cellulose with the unactivated glucocorticoid–receptor complexes, as well as another heavily phosphorylated species which was eluted with 3 M KCl following elution of the unactivated receptor complexes, were both polynucleotides. In a related study, Housley and Pratt[35] demonstrated that when mouse L cells were incubated in the presence of [^{32}P]orthophosphate and then subjected to the same purification scheme, several labeled entities, including a RNase-sensitive, heavily phosphorylated species of 21 kDa, were detected in the final DEAE-cellulose eluate. A subsequent series of experiments revealed that the ^{32}P-labeled species which was eluted from DEAE-cellulose by a high salt gradient (0.5–1.0 M KCl) following elution of unactivated glucocorticoid–receptor complexes also incorporated [^{14}C]uridine *in vivo* and was stained with ethidium bromide which indicated an approximate size of 100 nucleotides [25]. The results of these experiments also indicated that the amount of this high-salt-eluting material, presumably RNA, was reduced, but not eliminated, in mock purifications which lacked receptor. Taken collectively these previous studies indicated that some RNA may co-purify with unactivated receptor complexes through all three purification steps, but the majority of the RNA that co-purified through the first two purification steps (affinity chromatography and gel filtration) was effectively separated from unactivated receptor complexes by the final anion exchange chromatography step.

The data presented in this report indicate that RNA does co-purify with unactivated receptor complexes through the first two purification steps (Fig. 2), but that the majority of the RNA is not receptor associated. This conclusion is based on the observation that the relative quantity, size and heterogeneity of the RNA eluted from the final DEAE-cellulose column following elution of the unactivated [^{3}H]triamcinolone acetonide receptor complexes are essentially the same as for the RNA eluted after a mock purification which is devoid of unactivated receptor complexes (Figs 1 and 2). The RNA eluted from the DEAE-cellulose column in both the normal and mock purifications appears to be degraded (Fig. 2) and relatively nonspecific. The latter conclusion is evidenced by the fact that it is qualitatively very similar to total rat liver RNA, based on the hybridization patterns both with total hepatic RNA (Fig. 3) and with total genomic DNA (Fig. 4). Although these experiments directly address the question of specificity, they do not rule out the possibility that there may be a small subset of RNA molecules eluted from DEAE-cellulose which had been specifically associated with the unactivated form of the receptor. In that case the specific species could be masked by the numerous RNA species which are not receptor associated.

Although the majority of the RNA eluted from DEAE-cellulose by high-salt concentrations appears not to have been receptor associated, it co-purified with the unactivated glucocorticoid–receptor complexes through two fractionation steps, affinity chromatography and gel filtration. The affinity resin was washed extensively with buffer after being incubated with crude cytosol, but apparently this did not remove all cytosolic RNA, which may have been associated directly with the resin by an unknown mechanism. Published reports do exist which indicate that some steroid molecules can bind, via formation of hydrogen bonds as well as hydrophobic forces, to ribo- and deoxyribopolynucleotides under equilibrium conditions in aqueous buffers. Thus it is possible that cytosolic RNA could bind to the deoxycorticosterone molecules on the resin and that this RNA could subsequently be displaced by the 2 μM [^{3}H]TA which is added to elute the unactivated receptors from the resin. Once eluted from the affinity resin the RNA could potentially aggregate and hence co-elute from Bio-Gel A-1.5 m with unactivated [^{3}H]TA complexes with an apparent size of 300 kDa. This RNA would subsequently be separated from the unactivated receptor complexes based on different elution patterns from DEAE-cellulose. Another possibility which we have considered is that, although the majority of the RNA does not appear to be associated with the unactivated receptor protein, it may be complexed with other protein molecules which co-purify with the receptor through the affinity chromatography and gel filtration steps. In an attempt to address this possibility we digested the initial rat liver cytosol with protease K and then proceeded with our standard purification. Although electrophoretic analysis of the DEAE-cellulose eluate indicated the absence of both intact proteins and immunoreactive material detected with the BUGR 2 receptor monoclonal antibody, RNA was still recovered in the 1 M KCl eluate (data not shown).

In conclusion, it is important to note that RNA can be eluted from the final anion exchange column with salt concentrations both equivalent to [1, 25, 34], and significantly higher than [25, 33, present study], that required to elute purified unactivated receptor complexes. The present experiments do not address the possibility that the very small fraction of RNA which is eluted from DEAE-cellulose in the third step of our protocol, in exactly the same fractions as the unactivated receptor complexes, may be receptor-associated and relatively specific. Sablonniere *et al.*[36] have recently detected three RNA species (120, 100 and 80 nucleotides in size) in preparations of highly purified unactivated rat hepatic glucocorticoid–receptor complexes and have reported that only the largest of these species appears to be specifically associated with the receptor. However, the very low yield of this RNA would make hybridizations, such as those described in this report, very difficult. The fact that the bulk of the RNA which co-purified with the unactivated receptor complexes appears to be nonspecific obviously does not rule out a possible association of unactivated receptor complexes with specific RNA species *in vivo*. Data reported here, however, demonstrate that nonspecific RNA can copurify with the receptor through selective fractionation steps. Thus caution must be exercised when conclusions concerning receptor subunit structure are based on characterization of macromolecules present even in highly purified preparations.

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