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RNASE A EFFECTS ON SEDIMENTATION AND DNA BINDING PROPERTIES OF DEXAMETHASONE–RECEPTOR COMPLEXES FROM HeLa CELL CYTOSOL

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Summary—Dexamethasone–receptor complexes from HeLa cell cytosol sediment at 7.4S in low salt sucrose gradients, and at 3.8S in high salt gradients. If cytosol is heated at 25°C, receptor complexes sediment at 6.9S in low salt, and at 3.6S in high salt gradients. RNase A treatment of HeLa cell cytosol at 2°C results in receptor complexes which sediment at 6.9S in low salt gradients. RNase A treatment at 25°C, instead, results in receptor complexes which sediment in low salt gradients as two major forms at 6.5 and 4.8S. Receptor complexes from RNase A-treated cytosols sediment as their counterparts from untreated cytosols in high salt gradients. Although the shift in sedimentation properties of receptor complexes at 2°C is induced by RNase A, and not by other low molecular weight basic proteins or RNase T_1, the effect can be also obtained by inactive RNase A. The catalytically active enzyme, however, is required to observe 6.5 and 4.8S complexes after cytosol incubations at 25°C. Placental ribonuclease inhibitor prevents the appearance of RNase A-induced receptor forms at 25°C, but not at 2°C. Moreover, this inhibitor can prevent the 7.4 to 6.9S shift in sedimentation coefficient of receptor complexes caused by cytosol heating.

Dexamethasone–receptor complexes from HeLa cell cytosol show low levels of binding to DNA-cellulose, and heating at 25°C is required to observe a six-fold increase in DNA binding levels. RNase A treatment of cytosols at 2°C does not result in significant enhancement in receptor complex binding to DNA. If RNase A treatment is carried out at 25°C, however, DNA binding levels of receptor complexes increased by 25% over the values observed with control heated cytosol. This effect cannot be observed if RNase T_1 substitutes for RNase A. Placental ribonuclease inhibitor can prevent the temperature-dependent increase in DNA binding properties of dexamethasone–receptor complexes either in the presence or absence of exogenous RNase A.

These findings indicate that exogenous RNases can perturb the structure of dexamethasone–receptor complexes without being involved in the transformation process.

INTRODUCTION

In the proposed mechanism of steroid hormone action, the interaction of a steroid with its receptor protein is followed by a transformation of steroid–receptor complexes that enables them to bind tightly to chromatin in target cell nuclei [1]. The transformation of cytosolic steroid–receptor complexes to their nuclear, or DNA binding form, appears to be induced, *in vitro*, by several treatments which may involve heat [2, 3], salts [4, 5], dilution [6, 7], or gel filtration [7]. It has not been firmly established, however, the molecular mechanism(s) responsible for steroid–receptor complex transformation to the nuclear binding form. One hypothesis involves removal of RNA interacting with untransformed receptor complexes [8–11].

We here report the effects of RNase A on sedimentation, and DNA binding properties of dexamethasone–receptor complexes from HeLa cell cytosol, and show that the temperature-dependent transformation of receptor complexes to a DNA binding form is prevented, *in vitro*, by placental ribonuclease inhibitor.

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EXPERIMENTAL

Materials

[6,7-^{3}H(N)]Dexamethasone (46 Ci/mmol), and [5,6-^{3}H]Uridine (40.5 Ci/mmol) were purchased from New England Nuclear. CNBr-activated Sepharose 4B was obtained from Pharmacia. Aldolase, RNase A, RNase S-protein, RNase T_1, human placental ribonuclease inhibitor, porcine IgG, ovalbumin, bovine serum albumin, lysozyme, and cytochrome *c*, were obtained from Sigma. All other reagents were of analytical grade.

Cell culture and isotope labeling

HeLa S_3 cells were grown in suspension culture in Joklik-Modified Minimum Essential Medium (Gibco) in the presence of 5% newborn calf serum (Gibco), 75 U/ml penicillin, and 5 μg/ml streptomycin sulfate. Cells in logarithmic growth were harvested by low speed centrifugation, washed once with culture medium, and dispersed at a concentration of 2–4 × 10^6 cells/ml in the medium containing 5% charcoal-stripped [12] newborn calf serum. Cells were then incubated for 2 h at 2°C with 50 nM radioactive dexamethasone to allow formation of dexamethasone–receptor complexes. At the end of the incubation, cells were recovered by low speed

centrifugation, washed three times with a solution containing 20 mM Tris–HCl, pH 7.5 at 2°C, 0.15 M NaCl, 3 mM $MgCl_2$ (buffer A), and were then dispersed in a solution containing 10 mM Tris–HCl, pH 7.5 at 2°C, 10 mM NaCl, 1.5 mM $MgCl_2$ (buffer B). After 20 min swelling at 2°C, cells were broken with a Dounce homogenizer, and the suspension was then centrifuged for 15 min at 15,000 *g*. The supernatant was then centrifuged for 1 h at 105,000 *g* to obtain a crude cytosol, which was treated with a Dextran-coated charcoal pellet to remove free steroid. The charcoal pellet was obtained by centrifuging 1 vol (with respect to crude cytosol) of a stock solution composed of buffer B, and containing 0.5% (w/v) charcoal (Norit A), 0.05% (w/v) Dextran T 70 (Pharmacia), for 8 min at 1000 *g*. Charcoal treatment was carried out at 2°C for 10 min with occasional stirring, and charcoal-treated cytosol was obtained by centrifugation for 10 min at 8000 *g*.

Cytosol treatments

If not stated otherwise, charcoal-treated cytosols were incubated either at 2°C for 1 h, or at 25°C for 20 min. Additions were made by concentrated stock solutions in order to avoid excessive dilution of cytosols. Stock solutions of RNase A were incubated for 10 min at 100°C to inactivate proteases or DNases before use. These RNase A solutions did not show detectable protease or DNase activities. The concentration of ammonium sulphate in RNase T_1 stock solutions was lowered by centrifugal gel filtration [6]. In cytosol incubations involving RNase T_1, or placental RNase inhibitor, salts in the stock solutions contributed to the final ionic strength of incubation mixtures, and 1.5 M NaCl was used to reach a final 75 mM NaCl concentration in each sample. At the end of incubations, samples were immediately analyzed by DNA-cellulose binding assay and/or sucrose density gradient centrifugation.

DNA-cellulose binding

Transformation of dexamethasone–receptor complexes was evaluated by the DNA-cellulose centrifugation assay [13]. DNA-Cellulose was prepared as described by Alberts and Herrik[14], using calf thymus DNA and Whatman CF-11 powder. The final product contained 0.9–1.2 mg DNA/ml packed gel. Before assay, DNA-cellulose was washed extensively by suspension in buffer B and centrifugation for 8 min at 1000 *g*, to remove loosely associated DNA and fines. Cytosol samples were diluted 10-fold with buffer B, and aliquots in duplicate, or triplicate were incubated with either DNA-cellulose (200–300 μg of DNA), or CF-11 pellets, for 40 min at 2°C with occasional stirring. At the end of the incubation, samples were centrifuged for 10 min at 9000 *g*, and washed once with buffer B. The radioactivity retained by the gels was extracted by buffer B containing 0.6 M NaCl, recovered by centrifugation for 10 min at 9000 *g*, and determined. The radioactivity obtained from plain cellulose was subtracted from the amounts extracted from DNA-cellulose, to estimate specific binding of cytosolic dexamethasone–receptor complexes to DNA.

Sucrose density gradient centrifugation

Linear 5–20% (w/v) sucrose gradients in a solution composed of 20 mM Tris–HCl, pH 7.5 at 2°C, 1.5 mM EDTA, 5% (v/v) glycerol (buffer C), and either containing (high salt gradients), or lacking (low salt gradients) 0.3 M KCl, were employed to analyze dexamethasone–receptor complexes. Cytosol samples were layered on the top of the gradients, and centrifugation was performed with a Beckman SW-60 rotor in a Beckman L5-65 ultracentrifuge for 18 h at 50,000 rpm. Gradients were fractionated in 38 fractions, and the bottom of each tube was excised and included in the first fraction. Markers employed to determine sedimentation coefficients of dexamethasone–receptor complexes were aldolase (A, 7.9S), porcine IgG (IgG, 6.6S), bovine serum albumin (BSA, 4.4S), and ovalbumin (OV, 3.5S). Sedimentation coefficients of marker proteins were those listed by Sherman *et al.*[15]. Distances of marker proteins from the meniscus showed a standard deviation of less than 3% among different centrifugations. Sedimentation coefficients ($S_{20,w}$) of dexamethasone–receptor complexes were then assigned by the linear calibration plot derived from those external standards, according to the method of Martin and Ames[16].

Affinity chromatography of dexamethasone–receptor complexes

RNase A or lysozyme were coupled to CNBr-activated Sepharose. In a parallel incubation, the gel was treated in the absence of ligands to obtain a control gel. If not stated otherwise, the protein concentration of coupled gels was about 5 mg/ml of packed gel. Columns (0.5 ml of bed volume) were washed with a buffer (either buffer B, or C) containing 50 mM NaCl. HeLa cell cytosols were brought to a final 50 mM NaCl concentration, before being loaded onto the column. Unbound material was eluted with washing buffer, and the material retained by the gel was eluted, if not stated otherwise, by washing buffer containing 0.6 M NaCl. In the course of elutions, 4-drop fractions were collected, and assayed for radioactivity.

Determination of RNA hydrolysis

HeLa cells in logarithmic growth were incubated with 1 μCi of tritiated uridine/ml for 24 h at 37°C, harvested, and incubated for 2 h at 2°C with 50 nM unlabeled dexamethasone, before being processed to obtain DCC-treated cytosols. Cytosolic extracts were then incubated in the presence, or absence of RNase A. At the end of incubations, radioactive RNA was recovered, and hydrolyzed, as described by Chong and Lippman[10]. The soluble material thus obtained

was assayed for radioactivity, to estimate the amounts of RNA remaining in the samples.

Other methods

Radioactivity was measured in a Packard liquid scintillation spectrometer, with a toluene-based liquid scintillation fluid, as previously described [13]. The counting efficiency was about 35%. Protein was determined by the method of Lowry *et al.*[17] with bovine serum albumin as the standard. DNA was measured by the diphenylamine test, with calf thymus DNA as the standard [18]. Results shown in figures have been taken from one of the three or more replicate experiments performed.

RESULTS

Effects of RNase A on sedimentation properties of dexamethasone–receptor complexes from HeLa cell cytosol

In agreement with results obtained by others [19], dexamethasone–receptor complexes from HeLa cell cytosol sedimented at 7.4 ± 0.2S ($n = 19$) in low salt sucrose gradients, and at 3.8 ± 0.05S ($n = 5$) in high salt sucrose gradients (Fig. 1A). If HeLa cells were incubated with radioactive dexamethasone, and in the presence of 100-fold molar excess of unlabeled steroid, 7.4 and 3.8S peaks disappeared (not shown). Under our conditions, therefore, specific dexamethasone binding to glucocorticoid receptor was detected.

Incubation of HeLa cell cytosol at 2°C with 50 μg of RNase A/ml, led to a shift in sedimentation coefficient of dexamethasone–receptor complexes to 6.9 ± 0.3S ($n = 11$) in low salt sucrose gradients (Fig. 1A). This shift could be observed after cytosol treatment with RNase A concentrations higher than 5 μg/ml, but it could not be further affected by increasing either RNase A concentrations up to 2 mg/ml, or the time of treatment up to 24 h. In high salt sucrose gradients, sedimentation patterns of dexamethasone–receptor complexes in RNase A-treated cytosols were substantially the same as in control cytosol (Fig. 1A).

When HeLa cell cytosol was heated at 25°C, dexamethasone–receptor complexes sedimented at 6.9 ± 0.2S ($n = 23$), and some aggregated material appeared at the bottom of low salt sucrose gradients (Fig. 1B). In high salt gradients, however, aggregates could not be observed, and receptor complexes sedimented at 3.6 ± 0.04S ($n = 5$). Incubation of cytosol with RNase A at 25°C resulted in a marked change in sedimentation pattern of receptor complexes (Fig. 1B). Under these conditions, two major peaks could be detected at 6.5 ± 0.2S, and at 4.8 ± 0.3S ($n = 15$), and radioactivity sedimenting at the bottom of the gradient was low. Although this pattern could be detected as early as after 10 min of incubation, dexamethasone–receptor complexes could not be completely converted to the 4.8S form by increasing either the time of incubation up to 90 min, or RNase A concentrations up to 2 mg/ml. The levels of 4.8S complexes, instead, appeared to be inversely related to the protein concentration of cytosol extracts (Fig. 2). When RNase A-treated cytosols were analyzed by high salt sucrose gradients, dexamethasone–receptor complexes sedimented at 3.6 ± 0.05S ($n = 4$).

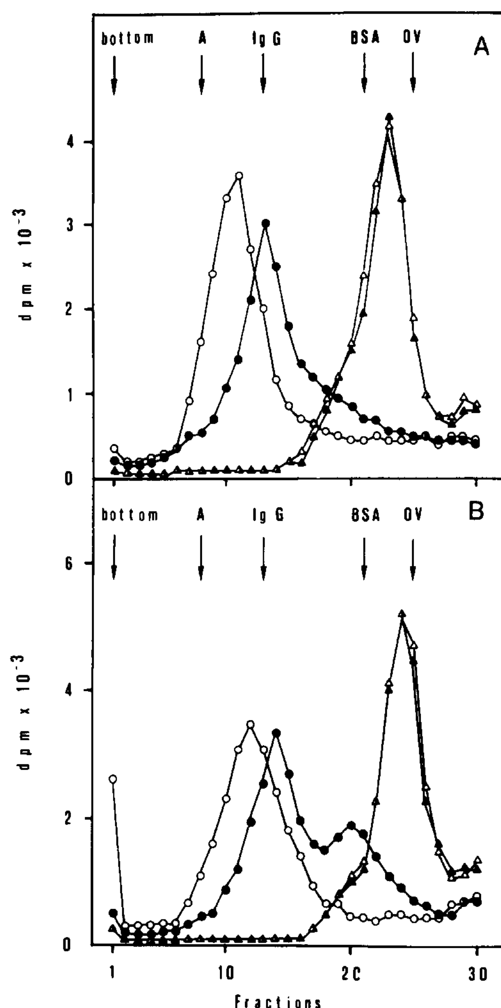

Fig. 1. Sucrose density gradient centrifugation of dexamethasone–receptor complexes from RNase A-treated and untreated HeLa cell cytosol. Cytosols were incubated at either 2°C for 1 h (A), or at 25°C for 20 min (B), and in the presence (●, ▲), or absence (○, △) of RNase A (50 μg/ml). Aliquots of samples were then analyzed by sucrose density gradient centrifugation in the presence (▲, △), or absence (●, ○) of 0.3 M KCl.

Since sedimentation of dexamethasone–receptor complexes in high salt gradients did not appear to be affected by RNase A addition to HeLa cell cytosols at both 2 and 25°C, the effects we observed could not be due to proteases contaminating RNase A preparations.

Effects of RNase A on binding of dexamethasone–receptor complexes to DNA–cellulose

When HeLa cell cytosol was maintained at 2°C,

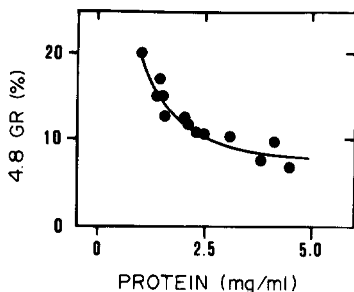

Fig. 2. Effect of protein concentration of HeLa cell cytosols on the levels of dexamethasone–receptor complexes sedimenting at 4.8S. HeLa cell cytosols were incubated for 20 min at 25°C with RNase A (50 μg/ml), before being subjected to low salt sucrose density gradient centrifugations. The percentage of dexamethasone–receptor complexes (GR) sedimenting at 4.8S was estimated by the ratio of radioactivity in fractions 19–20 over the radioactivity sedimenting in fractions 10–24 (total receptor complex).

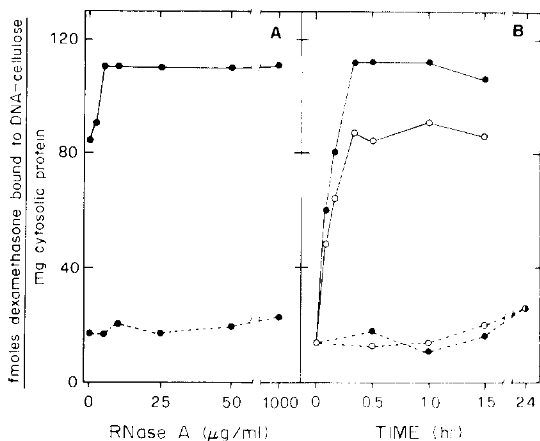

Fig. 3. Effect of RNase A on binding of dexamethasone–receptor complexes to DNA-cellulose. (A) HeLa cell cytosols were incubated either at 2°C for 1 h (dashed line), or at 25°C for 20 min (solid line), and in the presence of the indicated amounts of RNase A, before being subjected to DNA-cellulose binding assay. (B) HeLa cell cytosols were incubated for the indicated times at either 2°C (dashed line), or 25°C (solid line), and in the presence (●), or absence (○) of RNase A (50 μg/ml), before being subjected to DNA-cellulose binding assay.

binding of dexamethasone–receptor complexes to DNA–cellulose was low (Fig. 3A). Cytosol incubations in the presence of increasing RNase A concentrations did not lead to any enhancement of receptor complex binding to DNA. If temperature was raised to 25°C, DNA binding of receptor complexes in control cytosols showed a 6-fold increase over basal levels (Fig. 3A). Addition of RNase A to HeLa cell cytosol during incubation at 25°C, led to a further enhancement in receptor complex binding to DNA of about 25% with respect to control cytosol (Fig. 3A). As judged by sucrose density gradient centrifugations, the radioactive material retained by DNA-cellulose, and extracted by NaCl, represented dexamethasone-receptor complexes.

The time-course of dexamethasone–receptor complex transformation to the DNA binding form is shown in Fig. 3B. Maximal levels of receptor complex binding to DNA-cellulose were observed within 20 min of incubation at 25°C. Addition of RNase A to HeLa cell cytosol did not affect the time-course of receptor complex transformation, although it caused a 25% enhancement in receptor complex binding to DNA over control cytosol. When HeLa cell cytosols were aged for up to 24 h at 2°C, and either in the presence, or absence of RNase A, a significant enhancement of DNA binding could not be observed (Fig. 3B).

It has been proposed that removal of RNA could be involved in transformation of steroid–receptor complexes to a form able to interact with DNA [8–11]. The lack of effects we observed upon RNase A treatment of cytosols at 2°C could be due, therefore, to uncomplete hydrolysis of RNA. HeLa cells were thus incubated with tritiated uridine, and the extent of gross RNA hydrolysis was evaluated in cytosol preparations, and under our experimental conditions. When we measured the radioactivity obtained from perchloric acid-insoluble material of control and RNase A-treated samples, however, the extent of RNA hydrolysis did not correlate with DNA binding properties of dexamethasone–receptor complexes (Table 1). In fact, heating HeLa cell cytosols at 25°C, and in the absence of RNase A, was not accompanied by detectable decreases in perchloric acid-insoluble radioactivity. Moreover, the amounts of RNA remaining in cytosols treated with RNase A, were essentially the same after incubations either at 2°C for 24 h, or at 25°C for 20 min. These data could not rule out the possibility that RNA(s) had to be removed in the transformation of dexamethasone–receptor complexes. They indicated, however, that RNase A effects on DNA binding properties of receptor complexes, under our experimental conditions, were not simply due to the extent of RNA hydrolysis.

As we have shown, RNase A prevented aggregation of receptor complexes at 25°C (Fig. 1B). Enhancement of dexamethasone–receptor complex

Table 1. Effect of RNase A on RNA levels in HeLa cell cytosol

Cytosol treatment	RNase A	RNA (%)
None	–	100
20 min at 25°C	–	104 ± 2.5
	+	36.8 ± 2.8
24 h at 2°C	–	100 ± 2.8
	+	35.2 ± 0.8

HeLa cells were incubated with tritiated uridine for 24 h at 37°C. Cytosols were incubated as indicated, and in the presence, or absence of RNase A (50 μg/ml). Protein concentration in cytosol preparations varied between 2.2 and 4.6 mg/ml. Values represent means ± SD of three separate determinations, and 100% is 109 ± 2.1 pmol uridine released/mg cytosolic protein.

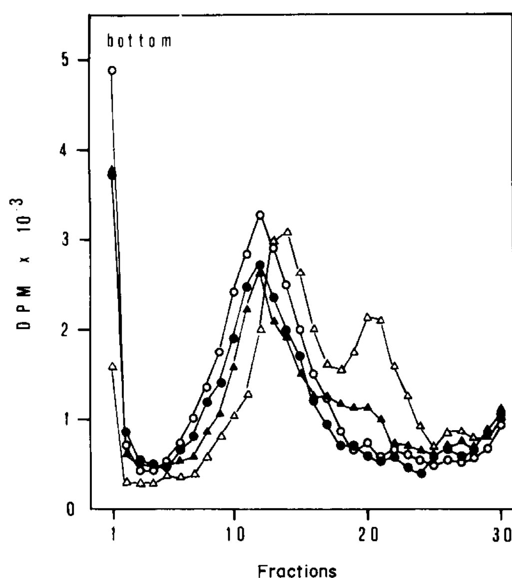

Fig. 4. Effect of RNase T_1, and lysozyme, on sedimentation of dexamethasone–receptor complexes from HeLa cell cytosol. Cytosol was incubated for 20 min at 25°C, and in the presence of RNase A (△), RNase T_1 (▲), lysozyme (●), or in the absence of additions (○). Enzymes were at a final 50 μg/ml concentration. At the end of the incubation, samples were subjected to low salt sucrose density gradient centrifugation.

binding to DNA-cellulose, therefore, could simply reflect a decrease in the receptor complex pool available to interaction with DNA. As judged by low salt sucrose gradient centrifugation, aggregated radioactivity was actually discarded during washing of DNA-cellulose. Since cytosol tratment with RNase A did not result in detectable changes in the levels of aspecific binding to DNA-cellulose, the data we obtained were replotted as the ratio between the radioactivity retained by the gel, and radioactive receptor complexes which did not aggregate at the bottom of low salt sucrose gradients. By this procedure, the fraction of the complexes retained by DNA-cellulose did not significantly differ in control and RNase A-treated cytosols (not shown). These results, therefore, suggested that, under our experimental conditions, the ability of RNase A to prevent aggregation of receptor complexes could be responsible for the effect on their DNA binding levels. This conclusion could be also supported by the finding that incubations of HeLa cell cytosols at 25°C with RNase T_1 did not cause an increase in DNA binding of receptor complexes.

Effect of RNase T_1 and inactive RNase A on sedimentation properties of dexamethasone–receptor complexes from cytosols incubated at 25°C

It has been shown that RNase A can interact with cytosolic estrogen receptor [20, 21]. That finding led us to ascertain whether the RNase A-induced shifts in sedimentation properties of dexamethasone–receptor complexes we observed might have been due to the net charge or size of the enzyme, rather than to its catalytic properties. We incubated, therefore, HeLa cell cytosols at 25°C, and in the presence of low molecular weight basic proteins. Under these conditions, neither lysozyne (Fig. 4), nor cytochrome *c* (not shown) could affect sedimentation properties of dexamethasone–receptor complexes. These results, therefore, indicated that the physicochemical properties of RNase A could not explain the shifts in sedimentation properties of receptor complexes induced by this enzyme at 25°C.

To test whether RNase A effects depended on its catalytic activity, HeLa cell cytosols were incubated with active, and inactive RNase A, or with RNase T_1. Incubation of cytosols at 25°C with RNase T_1, did not cause appearance of the 6.5S complexes observed after RNase A treatment, and receptor complexes sedimented as a major peak at 6.9S (Fig. 4). At a concentration of 50 μg/ml, however, RNase T_1 led to some accumulation of 4.8S complexes. The addition of RNase S-protein, an RNase A fragment devoid of ribonuclease activity [22], to HeLa cell cytosol at 25°C, did not result in the appearance of 6.5 and 4.8S complexes, and dexamethasone–receptor complexes sedimented at 6.9S (not shown). When cytosols were heated in the presence of both RNase A, and placental ribonuclease inhibitor, the appearance of 6.5 and 4.8S complexes was prevented, and dexamethasone–receptor complexes sedimented at about 6.9S (Fig. 5A). These results showed that appearance of 6.5 and 4.8S complexes required catalytically active RNase A, and that 4.8S complexes could be generated by both RNase A and RNase T_1. Only the former enzyme, however, was able to convert receptor complexes to the 6.5S form.

In the course of the aforementioned experiments, we noted that dexamethasone–receptor complexes from cytosols heated in the presence of placental ribonuclease inhibitor, but in the absence of RNase A, sedimented at about 7.4S (Fig. 5A). This observation suggested that placental RNase inhibitor could prevent the 7.4 to 6.9S conversion of dexamethasone–receptor complexes observed upon heating cytosols (Fig. 1). To directly test this hypothesis, cytosols were incubated at both 2 and 25°C, and in the presence or absence of RNase inhibitor (Fig. 5B). The results we obtained confirmed that contention, and treatment of HeLa cell cytosols at both 2 and 25°C with RNase inhibitor resulted in the detection of dexamethasone–receptor complexes sedimenting at about 7.3S (Fig. 5B). The addition of the inhibitor after cytosol incubation at 25°C could not reverse the shifts in sedimentation properties of receptor complexes in both control and RNase A-treated samples.

Effect of placental ribonuclease inhibitor on DNA binding properties of dexamethasone–receptor complexes

Since placental ribonuclease inhibitor could pre-

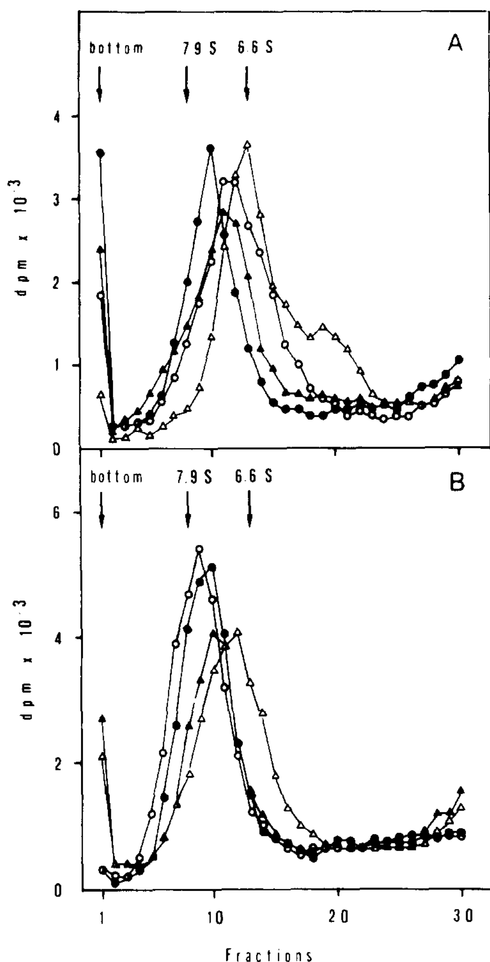

Fig. 5. Effect of placental ribonuclease inhibitor on sedimentation of dexamethasone–receptor complexes from HeLa cell cytosol. (A) Cytosols were incubated at 25°C for 20 min with (▲, △), or without (●, ○) RNase A (50 μg/ml), and in the presence (●, ▲), or absence (○, △) of placental ribonuclease inhibitor (1,500 units/ml). (B) Cytosols were incubated for 20 min either at 2°C (●, ○), or at 25°C (▲, △), and in the presence (●, ▲), or absence (○, △) of placental ribonuclease inhibitor (1500 units/ml). At the end of the incubations, samples were subjected to low salt sucrose density gradient centrifugation. Aldolase (7.9S), and porcine IgG (6.6S) were used as external standards in the gradients.

vent the temperature-induced shifts in sedimentation coefficient of dexamethasone–receptor complexes, we ascertained whether the inhibitor could also affect DNA binding properties of receptor complexes. HeLa cell cytosols were then incubated in the presence or absence of placental RNase inhibitor, before being subjected to DNA-cellulose binding assay (Table 2). When this inhibitor was present during cytosol incubations at 25°C, transformation of dexamethasone–receptor complexes was substantially abolished, and their DNA binding levels were indistinguishable from the ones observed with unheated cytosols. The presence of RNase A together with placental RNase inhibitor during cytosol incubations, however, could not reverse the inhibitory effect of this compound, and DNA binding levels were only slightly higher than the ones observed with unheated cytosols (Table 2). Moreover, addition of the inhibitor to heat treated cytosols before DNA binding assay did not affect the levels of dexamethasone–receptor complexes retained by the gel, and identical amounts of complexes were extracted by 0.6 M NaCl (not shown). Placental ribonuclease inhibitor, therefore, could prevent *in vitro* transformation of dexamethasone–receptor complexes, without interfering with their binding to DNA-cellulose.

The RNase A effect at 2°C does not depend on its catalytic activity

By DNA-cellulose binding assay, we could establish that receptor complexes in heat-treated cytosols could associate to DNA, whereas this was not the case when cytosols were incubated either with RNase A at 2°C, or with RNase A plus placental RNase inhibitor at 25°C. Moreover, incubations of HeLa cell cytosols at 25°C in the presence of this inhibitor did not result in the appearance of 6.9S complexes in control samples, or 6.5 and 4.8S complexes in RNase A-treated cytosols (Fig. 5). In the latter case, we noted that receptor complexes sedimented at 6.9S, as we observed upon RNase A treatment of cytosols at 2°C (Fig. 1). Thus we speculated that recovery of a 6.9S receptor form in RNase A-treated samples might not depend on heating, and on a catalytically active enzyme. To test this hypothesis, HeLa cell cytosols were incubated at 2°C, and in the presence of active or inactive RNase A. As it is shown in Fig. 6A, the addition of placental RNase inhibitor did not affect the sedimentation pattern of dexamethasone–receptor complexes in both control and RNase

Table 2. Specific binding of dexamethasone–receptor complexes to DNA-cellulose after cytosol incubations in the presence of RNase A and placental ribonuclease inhibitor

Sample	Additions	DNA binding (%)
Heated cytosol	None	100
	RNase A (50 μg/ml)	122.4 ± 3.4
	RNase Inhibitor (1500 units/ml)	22.5 ± 1.3
	RNase A (50 μg/ml) + RNase Inhibitor (1500 units/ml)	30.7 ± 4.3
Unheated cytosol	None	18.3 ± 5.0

HeLa cell cytosols were incubated for 20 min at either 25 or 2°C, and in the presence of indicated additions. Values represent means ± SD of five separate determinations, and 100% is 92.3 ± 4.0 fmol dexamethasone bound to DNA-cellulose/mg cytosolic protein.

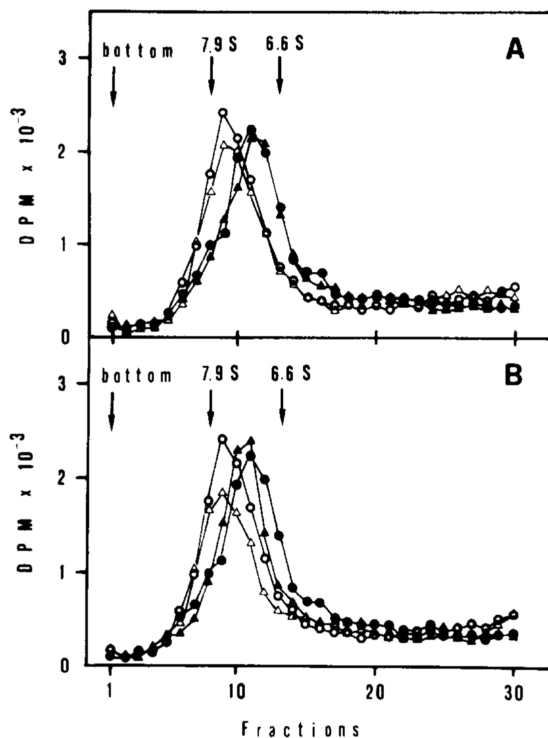

Fig. 6. Sucrose density gradient centrifugation of dexamethasone–receptor complexes from HeLa cell cytosol treated with active and inactive RNase A at 2°C. (A) Cytosol was incubated for 1 h at 2°C with (●, ▲), or without (○, △) RNase A (50 μg/ml), and in the presence (▲, △), or absence (●, ○) of placental ribonuclease inhibitor (1500 units/ml). (B) Cytosol was incubated for 1 h at 2°C without additions (○), or in the presence of RNase A (●), RNase S-protein (▲), or lysozyme (△), at a final 50 μg/ml concentration. At the end of the incubations, samples were subjected to low salt sucrose density gradient centrifugation. Aldolase (7.9S), and porcine IgG (6.6S) were used as external standards in the gradients.

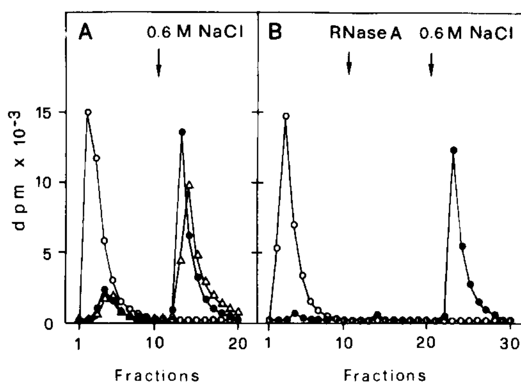

Fig. 7. Affinity chromatography of dexamethasone–receptor complexes from HeLa cell cytosol. (A) Portions of DCC-treated cytosol were brought to a final 50 mM NaCl concentration, and were applied to columns containing control Sepharose (○), RNase A-Sepharose (●), or lysozyme-Sepharose (△). After columns were washed with buffer B containing 50 mM NaCl, the retained material was eluted with buffer B containing 0.6 M NaCl. (B) Portions of DCC-treated cytosol were brought to a final 50 mM NaCl concentration, and were applied to columns containing control Sepharose (○), or RNase A-Sepharose (●). In this experiment, RNase A-Sepharose was mixed with control Sepharose, in order to have 0.5 μg RNase A/ml settled gel. After columns were washed with buffer B containing 50 mM NaCl, material retained by the columns was eluted with washing buffer containing 5 mg RNase A/ml, followed by buffer B containing 0.6 M NaCl.

A-treated samples. If, under the same conditions, cytosols were incubated with RNase S-protein, sedimentation pattern of receptor complexes did not significantly differ from those observed with RNase A-treated cytosols (Fig. 6B). Moreover, sedimentation patterns of lysozyme (Fig. 6B), or RNase T_1-(not shown) treated cytosols were essentially superimposable to the ones observed with control samples. These results indicated that appearance of 6.9S complexes in RNase A-treated cytosols did not depend on heating and on the catalytically active enzyme. Since lysozyme was without effect, appearance of 6.9S complexes of 2°C should not also depend on addition of low molecular weight basic proteins. We addressed, therefore, the question on whether, under our experimental conditions, RNase A might directly interact with dexamethasone–receptor complexes. To evaluate this possibility, we first employed affinity chromatography of HeLa cell cytosols by immobilized RNase A. Although receptor complexes could be retained by RNase A-Sepharose, and not by control Sepharose, dexamethasone–receptor complexes were also retained by lysozyme–Sepharose (Fig. 7A). The chromatographic behavior of dexamethasone–receptor complexes depended on the salt concentration of the buffer employed in our procedures, but it was not affected by either EDTA, or Mg^{2+}. In fact, the same elution patterns were observed when buffer C substituted for buffer B in the course of affinity chromatography.

Under our chromatographic conditions, both lysozyme, and RNase A should be positively charged. If we carried out DEAE-cellulose chromatography of unheated cytosols, dexamethasone–receptor complexes were eluted as a major peak at 120 mM NaCl, and this salt concentration could also elute more than 80% of radioactive complexes retained by RNase A-Sepharose (not shown). The chromatographic behavior of receptor complexes under affinity chromatography, therefore, could simply reflect the capacity of untransformed complexes to interact with anion exchangers [19, 23, 24]. The contention that receptor complex binding to RNase A-Sepharose should not be specific, was also supported by the finding that a 10,000-fold molar excess of RNase A could not lead to a significant elution of receptor complexes retained by RNase A-Sepharose (Fig. 7B). Moreover, by low salt sucrose gradient centrifugations, we could not observe increased RNase activity cosedimenting with receptor complexes from RNase A-treated cytosols, with respect to control cytosols (not shown).

It seems unlikely, therefore, that RNase A effect on dexamethasone–receptor complexes at 2°C might be simply explained by a direct interaction of the enzyme with receptor complexes.

DISCUSSION

In line with results obtained in other experimental systems involving steroid–receptor complexes [8, 11, 20, 21, 25], RNase A could perturb sedimentation properties of dexamethasone–receptor complexes from HeLa cell cytosol. The appearance of 6.9S complexes at 2°C, and 6.5 and 4.8S complexes at 25°C, however, might not have been caused by a common mechanism because a catalytically active enzyme was required to obtain the effects at 25°C, whereas this was not the case when incubations were carried out at 2°C. Moreover, both RNase A and RNase T_1 could lead to appearance of 4.8S complexes, but this phenomenon could not be simply related to RNA hydrolysis. If this were the case, cytosol treatment with high amounts of RNase, and for a long time, should cause a complete conversion of receptor complexes to the 4.8S form. This could not be obtained, however, and the amounts of this receptor form appeared to depend on the protein concentration of our samples, but not to the extent of RNA hydrolysis.

Others have investigated RNase effects on sedimentation properties of steroid–receptor complexes. The RNase-induced appearance of receptor complexes sedimenting at about 4S we observed, has been reported in some systems [8, 11, 25], but not in others [10, 35]. Among the former studies, RNase A could completely convert receptor complexes in the 4S form only in one case [25]. Moreover, it has been shown that RNase A can directly interact with estrogen–receptor complexes, leading to a change in their sedimentation properties [20, 21]. Thus, RNase effects on sedimentation properties of steroid–receptor complexes cannot be only related to the catalytic properties of the enzyme(s). In the present study, for instance, the most likely interpretation of RNase effects on sedimentation properties of dexamethasone–receptor complexes appears to be that, depending on the conditions employed in experimental procedures, these enzymes could affect the structure of receptor complexes by either their catalytic properties, or their physicochemical characteristics. Since it has been proposed that, under low salt conditions, glucocorticoid–receptor complexes could be associated in multimeric structures [19, 23, 26–28], RNases could perturb the association among dexamethasone–receptor monomers in our cytosol preparations. The molecular mechanism(s) by which RNases can bring about their effects, however, remains to be elucidated.

Under our experimental conditions, RNA could interact with receptor complexes [10, 11, 13, 25, 29–34]. In the course of this investigation, we did not directly study the possible association of RNA to cytosolic dexamethasone–receptor complexes. Placental ribonuclease inhibitor, however, could block the decrease in sedimentation coefficient of receptor complexes detected after cytosol heating. These data, therefore, suggest that dexamethasone–receptor–RNA complexes could be present in HeLa cell cytosol.

By the use of exogenous RNases, it has been proposed that transformation of steroid–receptor complexes to the DNA binding form might involve removal of RNA [8–11]. This contention has been originally suggested by the finding of RNase A effects on sedimentation properties of glucocorticoid–receptor complexes [8]. It has been later shown that cytosol treatment with RNases could lead to an increase in the amounts of steroid–receptor complexes bound to DNA [10, 11]. This effect on receptor complex transformation has been obtained with either RNase A, or T_1, and it was reported to occur at 25–30°C [10, 11]. An increase in dexamethasone–receptor complex binding to DNA-cellulose could be also observed after we treated HeLa cell cytosol at 25°C with RNase A, but not with RNase T_1. A direct involvement of exogenous RNases in transformation process, however, could not be deduced by the findings we obtained. On the one hand, RNase A could not transform cytosolic receptor complexes at 2°C in spite of extensive hydrolysis of RNA. On the other hand, a lack of correlation was observed between sedimentation, and DNA binding properties of dexamethasone–receptor complexes in RNase-treated and untreated samples. The appearance of 6.5 and 4.8S complexes, in fact, was not mandatory to observe high levels of receptor complex binding to DNA–cellulose. Moreover, although 6.9S complexes of control heated cytosols efficiently bound to DNA-cellulose, receptor complexes with the same sedimentation coefficient, and generated by RNase A at 2°C, or detected in cytosols heated in the presence or RNase A plus placental RNase inhibitor, were not transformed to their DNA binding form. Tymocyzko and Phillips have also shown that placental RNase inhibitor can affect the levels of glucocorticoid–receptor complex able to bind to DNA [11]. In that study, the inhibitor could prevent the increase in receptor complex binding to DNA caused by RNase A, but not by heating. Placental ribonuclease inhibitor, under our experimental conditions, could also prevent the heat-induced transformation of dexamethasone–receptor complexes in the absence of exogenous RNases. Thus, our observations suggest that *in vitro* transformation of dexamethasone–receptor complexes to the DNA binding form, under low ionic strength conditions, involves an RNase-sensitive step. Our data cannot exclude, however, the possibility that placental ribonuclease inhibitor might directly interfere with transformation process by some mechanism unrelated to inhibition of RNA hydrolysis.

If transformation involves an RNase-sensitive step,

this should represent hydrolysis of some RNA associated to receptor complexes by an RNase present in HeLa cell cytosol. This conclusion, however, does not imply that RNA removal can be exhaustive in a description of transformation process *in vitro*. Thus, RNA hydrolysis might represent a primary event which enables receptor complexes to be transformed by a second process. For instance, RNA hydrolysis might cause a change in the structure of receptor complexes which could expose the region of receptor proteins involved in transformation. In any case, cytosol heating appears to be required to observe transformation of dexamethasone–receptor complexes under our low ionic strength conditions. The supposed RNase of HeLa cell cytosol, therefore, should be unable to bring about its effect under low temperature.

Other studies are required before the hypothesis on RNA removal from steroid–receptor complexes can be extrapolated to account for receptor complex transformation *in vivo*.

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