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# RNA INDUCED REVERSAL OF GLUCOCORTICOID RECEPTOR ACTIVATION

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SUMMARY: The combination of partially purified, activated (nuclear binding) mammary glucocorticoid receptor with a macromolecule present in cytosol converts the receptor to a form with properties of native receptor. After salt activation of native receptor and subsequent purification by DEAE-cellulose chromatography, the activated receptor sediments in sucrose density gradients at 4.0S and binds (80-90%) ATP-agarose. Combination of this partially purified, activated receptor with a factor present in crude cytosol allows the receptor to sediment in the position of native receptor (8.4S). Furthermore, this receptor form, like native receptor, does not interact significantly with ATP-agarose. The cytosolic factor responsible for this receptor conversion is macromolecular (MW > 5000) and does not interact with ATP-agarose. It retains its transformation reversal properties after incubation at 100°C for 10-15 minutes in the presence and absence of 8M urea/1% SDS/1mM DTT and is not destroyed by treatment with DNase. However, the receptor transformation reversal factor is inactivated by treatment with RNase. These results demonstrate that glucocorticoid receptor activation is a reversible process and suggest that RNA is involved.

## INTRODUCTION

The molecular mechanism of steroid hormone receptor activation is an area of intensive investigation at the present time. Activation is usually defined as an 'alteration' in the cytoplasmic receptor protein which takes place rapidly in vivo upon its interaction with a specific steroid (1) and results in the transformation of a steroid-receptor complex with little or no affinity for nuclei to one which has a greatly increased affinity for nuclei (1,2). Generally assumed to be a necessary step in steroid hormone action, receptor transformation has been extensively studied in vitro. However, because of the difficulties associated with the isolation and purification of nuclei for determinations of receptor activation in vitro, several investigators have carefully developed more defined and less cumbersome biochemical assays to test for this event

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(1-4). These alternative methods take advantage of certain well characterized changes in the receptor molecule which have been shown to accompany activation. For the glucocorticoid receptor, these changes include a striking decrease in apparent molecular weight[2] (5) and an altered elution profile from ion exchange resins (both anionic and cationic) (1,2,4,6).

During our investigation into the molecular mechanism of glucocorticoid receptor activation and its prevention by molybdate, certain observations made it necessary for us to determine whether activation is a reversible process. In particular, we wished to establish whether activated receptor could be converted back to its nonactivated or native state. In this preliminary communication, we report the use of glucocorticoid receptor from lactating goat mammary tissue to demonstrate the reversal of glucocorticoid receptor activation. We additionally present data implicating RNA as a constituent of the cystolic factor responsible for this conversion.

## Experimental Procedures

Materials: [$^3$H]dexamethasone (50 Ci/mmol) was purchased from New England Nuclear. Purified bovine liver catalase (11.3S), human gamma globulin (7.1S), bovine hemoglobin (4.4S), equine heart cytochrome c (1.9S), pancreatic RNase A (100 units/mg), DNase I (3,270 units/mg) and ATP-agarose (1.75 μmoles/ml of packed gel) were all obtained from Sigma Chemical. DEAE-cellulose (DE-52) was from Whatman. Sephadex G-25 (coarse) was obtained from Pharmacia Fine Chemicals. Crystalline RNase $T_1$ (Sankyo Co., Ltd., Tokyo, Japan) was obtained from Calbiochem-Behring Corp. and RNase B (2290 u/mg) was from Worthington Biochemical. All other reagents, including sodium dodecyl sulfate (SDS) and dithiothreitol (DTT), were of analytical reagent grade.

Methods: Mammary glands from lactating goats obtained fresh from a local slaughter house were cleaned, minced and stored in liquid nitrogen until use. Cytosol was prepared and labeled with 20nM [$^3$H]dexamethasone overnight for 6-18h at 0°C as described previously (7) using TDG buffer (10mM Tris-HCl, 1.0mM dithiothreitol and 25% glycerol (by volume), pH 7.4 at 0°C). In separate experiments, by Scatchard analyses, sucrose density gradient centrifugation and DEAE-cellulose chromatography, we have demonstrated the absence of nonsuppressible [$^3$H]dexamethasone binding sites in cytosol from lactating goat mammary tissue.[2]

Transformation of cytosolic glucocorticoid receptors from the native to the activated (nuclear binding) form was accomplished at 0°C by chromatography of the [$^3$H]dexamethasone labeled cytosol through Sephadex G-25 equilibrated with TDG buffer containing 0.3M KCl (TDGK). Receptor was incubated in the 0.3M KCl-containing buffer for 1-2 hr at 0°C before desalting by Sephadex G-25 chromatography. Activated glucocorticoid receptors were isolated by DEAE-cellulose chromatography essentially according to Sakaue and Thompson (4) with modifications as described elsewhere (6).

ATP-agarose (ATP attached through the ribose hydroxyls to beaded agarose via a six carbon spacer) contained approximately 2 μmoles of ATP per ml of settled gel. After washing with TDG buffer containing 1.0 M KCl, the gel was poured into a 1.3 cm diameter column and packed to a volume of 1.2 ml. The packed gel was washed with 10-20 ml of TDG buffer before use. Samples, previously labelled with [$^3$H]dexamethasone were applied to the column and incubated for 30 min before unbound radioactivity was eluted by washing with TDG buffer. Bound radioactivity was eluted with 1.0M KCl in TDG Buffer. Fractions of 1.0 ml each were collected and the radioactivity was determined as for DEAE-cellulose chromatography (6).

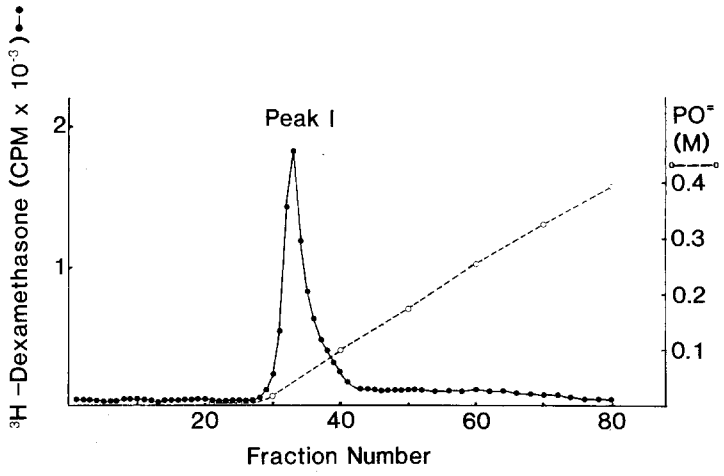


Figure 1: Isolation of activated (peak I) receptor from G-25/KCl treated cytosol by DEAE-cellulose chromatography. The activated receptor elutes at 0.05 M $PO_4^=$ (94% recovery) and was generated freshly for each experiment.

Immediately prior to sucrose density gradient analyses, [$^3$H]dexamethasone labeled cytosol (or partially purified receptor fractions) were passed through small (10-11ml) Sephadex G-25 columns at 0°C to remove free steroid or KCl. Two hundred microliters of these fractions were then layered onto linear 5-20% sucrose density gradients prepared in TDG buffer. The gradients were centrifuged, fractionated and analyzed for tritium as described previously (5). The linearity of representative gradients was confirmed and peaks of [$^3$H]dexamethasone binding activity were assigned sedimentation coefficients ($s_{20,w}$) as described previously (5).

## Results

Native (untransformed) [$^3$H]dexamethasone-receptor complexes in unfractionated cytosol were activated, desalted and chromatographed on DEAE-cellulose as described in Methods. As shown in Figure 1, the G-25/KCl activated receptor elutes from DEAE-cellulose as a single radioactive peak (peak I) early after initiation of the salt gradient (0.05M $PO_4^=$). Even though early elution from DEAE-cellulose has been a much demonstrated quality of activated glucocorticoid receptors from varied sources[2] (1,4,6, 8), the fully activated status of peak I mammary glucocorticoid receptor was additionally confirmed by sucrose density gradient centrifugation, ATP-agarose chromatography (3,6) and binding to isolated nuclei. These additional experiments were performed because of the important requirement that any experiments designed to examine reversibility of the transformation process must necessarily be performed with completely activated starting material. In contrast to native receptor which sediments in our gradients at 8.4 $\pm$ 0.4S (n=6), the partially purified, activated (peak I) receptor sediments at 4.0 $\pm$ 0.4S (n=9) under the same conditions. Sucrose density gradient centrifugation data from a

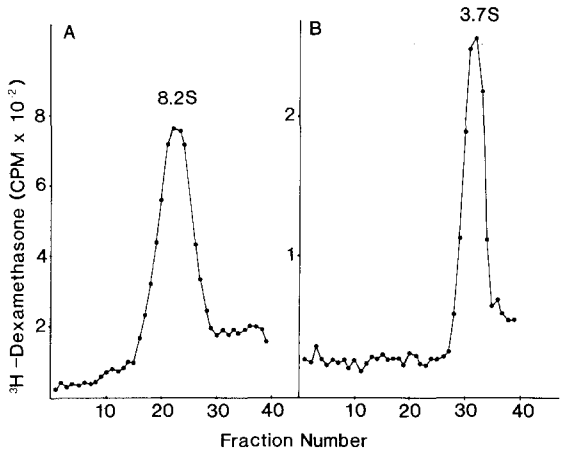


Figure 2: Sucrose density gradient centrifugation of (A) native cytosolic [$^3$H]dexamethasone-receptor complexes prepared and maintained in TDG buffer, and (B) partially purified, activated peak I receptor, also in TDG buffer. Receptors were assigned sedimentation coefficients as described previously (5). Note that peak I (activated) receptor sediments at 4S in the absence of salt.

representative experiment are illustrated for native and activated receptor in Figures 2A and 2B, respectively. Additionally, whereas native receptor shows low binding (13.9 $\pm$ 5.9%, n=8) to ATP-agarose, activated receptor (peak I) binds well (85.7 $\pm$ 8.2%, n=16). Finally, as expected, we have found that only receptor in the activated (peak I) state binds to isolated nuclei (data not shown). It is interesting to note that peak I (activated) receptor appears stable as such. The sedimentation profile (indistinguishable in high and low salt), DEAE-cellulose elution profile and ATP-agarose binding characteristics of isolated peak I have been examined and are unchanged by the presence of molybdate or by further transient exposure to elevated ionic strength (manuscript in preparation).

To study potential reversibility of the transformation (activation) process, the partially purified peak I receptor was titrated with crude cytosol which had been previously equilibrated with 2$\mu$M radioinert dexamethasone. We have determined that crude cytosol labeled in this manner with excess radioinert dexamethasone does not bind [$^3$H]dexamethasone which might have dissociated from peak I. Even when amounts of free [$^3$H]dexamethasone equivalent to that amount bound by peak I were incubated overnight (18 hr) at 0$^\circ$ C with radioinert dexamethasone labeled cytosol, no macromolecular bound radioactivity was detected in the cytosol (data not shown). Furthermore, the amount of [$^3$H]dexamethasone bound to peak I was not affected significantly by overnight incubation with the radioinert dexamethasone labeled cytosol (data not shown).

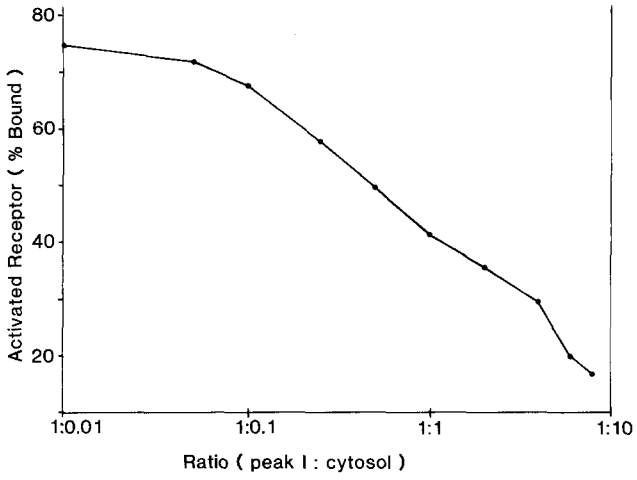


Figure 3: Titration of activated, peak I [$^3$H]dexamethasone-receptor complexes with increasing volumes of unfractionated cytosol containing 2μM radioinert dexamethasone. Ordinate represents [$^3$H]dexamethasone-receptor complexes bound (as percent of total activity added) after washing the column with 6-10 volumes of TDG buffer. Peak I alone and native (unactivated) [$^3$H]dexamethasone receptor complexes bound 83% and 13% respectively. Dilution of peak I with up to 10 volumes of buffer had no measurable effect on subsequent peak I binding to ATP-agarose. Samples were chromatographed immediately after mixing. Values presented are from one representative experiment.

As shown in Figure 3, the ATP-agarose binding ability of peak I could be diminished in a dose-dependent fashion by increasing volumes of cytosol to levels reminiscent of native (unactivated) receptor. Dilution of peak I with buffer alone did not signficantly alter its ATP-agarose binding properties. Furthermore, presaturation of the ATP-agarose columns with up to 7.5 volumes of cytosol did not diminish subsequent peak I binding (data not shown). By comparison, peak I mixed with 7.5 volumes of cytosol just prior to ATP-agarose chromatography bound only 13% (see Figure 3). These data indicate that cytosol contains a factor which apparently interacts directly with activated receptor to prevent its interaction with ATP-agarose.

To further examine the possibility that this factor was interacting directly with the partially purified activated receptor, peak I was combined with radioinert dexamethasone labeled cytosol and centrifuged through sucrose density gradients prepared with TDG buffer. Figure 4 demonstrates that the sedimentation coefficient of peak I receptor is dramatically altered. In contrast to activated (peak I) receptor which sedimented at 4.0 S under these same conditions (Figure 2B), peak I after recombination with crude cytosol sedimented at (8.22 $\pm$ 0.68 S, n = 6). This value does not differ

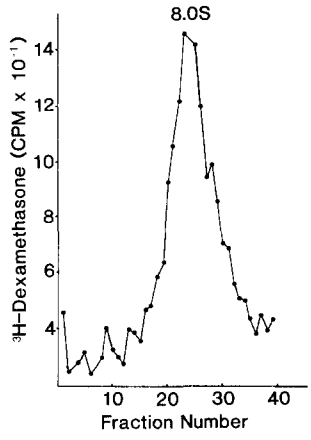


Figure 4: Altered sedimentation profile of activated (peak I) receptor resulting from its interaction with transformation reversal factor in crude cytosol. Peak I (activated) receptor was desalted by Sephadex G-25 chromatography, mixed with an equal volume of radioinert dexamethasone (2μM) labeled cytosol and immediately analyzed by sucrose density gradient centrifugation in TDG buffer as described for Figure 2.

significantly from that obtained for native receptor (8.4 ± 0.4S, n = 6) by Student's 't' test.

Preliminary characterization of the transformation reversal factor in crude cytosol preparations suggest that it is distinct from the low- and high-molecular weight translocation inhibitors and modulators described for other steroid receptor systems (9-14). The apparent molecular weight of the transformation reversal factor is greater than 5000 as it is excluded from Sephadex G-25. It is stable to 100° C (10-15 min) in the presence and absence of 8M urea/1%SDS/1mM DTT and to digestion with DNase I (10,000 units for 40 min at 25° C in the presence of 5mM $MgCl_2$). However, it can be inactivated by treatment with RNase A, B or $T_1$ (50-1000 units for 30-60 min at 25° C).

## Discussion

Transformation of steroid receptors to their activated (nuclear binding) form *in vitro* has been amply demonstrated (1-6). Recently, biochemical confirmation of the probable occurrence of activation *in vivo* has been reported (1). It appears that receptor activation and the equilibrium concentration of activated receptor may depend upon several factors in addition to the obligatory attachment of steroid (15). The data described here suggest that a macromolecular component (transformation reversal factor) present in unfractionated mammary cytosol can reverse activation and maintain an equilibrium in the direction of native receptor. We have shown that the influence of

transformation reversal factor on partially purified, activated peak I receptor is pronounced. The stability of this factor to treatments which are known to irreversibly denature native protein or degrade DNA structure, and its sensitivity to RNase treatment suggests that an RNA polymer is directly involved with the receptor. In support of this concept, Costello and Sherman have recently reported that the glucocorticoid receptor from mouse mammary tumor cytosol sediments at 5.3S and 8.7S when analyzed in 50mMKCl (16), however, after treatment of this cytosol with RNase, the relative amount of 5S receptor was increased. We have also observed an RNase dependent decrease in the sedimentation coefficient (8S→4S) of goat mammary glucocorticoid receptor which is accompanied by a marked increase in the ability of the receptor to bind ATP-agarose (manuscript in preparation). Other studies (17,18) have shown that RNA can inhibit the binding of activated steroid-receptor complexes to DNA. We feel that a nonenzymatic model for reversible receptor activation in vivo involving interaction of receptor with a non-steroid-binding, RNA containing entity is worthy of investigation. We are currently attempting to more completely characterize the transformation reversal factor(s) involved as well as the nature of its interaction with mammary glucocorticoid receptor.

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References

1. Munck, A., and Foley, R. (1979) Nature 278, 752-754.
2. Bailly, A., Le Fevre, B., Savouret, J.-F., and Milgrom, E. (1980) J. Biol. Chem. 255, 2729-2734.
3. Miller, J.B., and Toft, D.O. (1978) Biochemistry 17, 173-177.
4. Sakaue, Y., and Thompson, E.B. (1977) Biochem. Biophys. Res. Commun. 75, 533-545.
5. Hutchens, T.W., Markland, F.S., and Hawkins, E.F. (1981) Biochem. Biophys. Res. Commun. 103, 60-67.
6. Hutchens, T.W., Hawkins, E.F., and Markland, F.S. (1982) J. Steroid Biochem., in press.
7. Hawkins, E.F., Horn, D., and Markland, F.S., Jr. (1980) Cancer Res. 40, 2174-2178.
8. Markovic, R.D., Eisen, H.J. Parchman, L.G., Barnett, C.A., and Litwack, G. (1980) Biochemistry 19, 4556-4564.
9. Cake, M.H., Goidl, J.A., Parchman, L.G., and Litwack, G. (1976) Biochem. Biophys. Res. Commun. 71, 45-52.
10. Bailly, A., Sallas, N., and Milgrom, E. (1977) J. Biol. Chem. 252, 858-863.
11. Sato, B., Noma, K., Nishizawa, Y., Nakao, K., Matsumoto, K., and Yamamura, Y. (1980) Endocrinology 106, 1142-1160.
12. Izawa, M. (1979) Endocrinol. Japon. 26, 431-437.

13. Nishizawa, Y., Maeda, Y., Noma, K., Sato, B., Matsumoto, K., and Yamamura, Y. (1981) Endocrinology 109, 1463-1472.
14. Colvard, D.S. and Wilson, E.M. (1981) Endocrinology 109, 496-504.
15. Bailly, A., Savouret, J.-F., Sallas, N., and Milgrom, E. (1978) Eur. J. Biochem. 88, 623-632.
16. Costello, M.A. and Sherman, M.R. (1980) 62nd Annual Meeting of the Endocrine Society, Washington, D.C. (Abstract 399).
17. Liao, S., Smythe, S., Tymoczko, J.L., Rossini, G.P., Chen, C., and Hiipakka, R.A. (1980) J. Biol. Chem. 255, 5545-5551.
18. Feldman, M., Kallos, J., and Hollander, V.P. (1981) J. Biol. Chem. 256, 1145-1148.