BINDING OF RECEPTOR-HYDROCORTISONE COMPLEXES TO ISOLATED NUCLEI FROM EMBRYONIC NEURAL RETINA CELLS

P.K. Sarkar* and A.A. Moscona

Departments of Biology and Pathology and the
Committee on Developmental Biology, University of Chicago,
Chicago, Illinois 60637

Received February 5,1974

Summary: Hydrocortisone (HC) induces glutamine synthetase in the embryonic chick neural retina. The binding of cytoplasmic receptor-hydrocortisone (R-HC) complexes to isolated retina nuclei has been studied in a cell-free system. Optimal conditions, specificity and quantitative aspects of binding were determined. The isolated nuclei retained binding specificity for the R-HC complex prepared from retina cytosol. Free HC, estradiol-receptor complexes from retina cytosol and HC-receptor complexes from mouse brain cytosol or from chick serum did not bind to the nuclei. Assuming monovalency of the binding sites, the number of nuclear acceptor sites per retina cell for the R-HC complex was estimated to be in the range of 1500. These sites were resistant to RNAse but sensitive to DNAse.

Hydrocortisone (HC)** and several other 11-β-OH glucocorticoids induce glutamine synthetase (GS) in cultures of neural retina tissue of chick embryo by eliciting a transcription-dependent increase in the rate of synthesis of the enzyme (1-4). The interaction of steroids with target cells involves several steps (5-8): (a) binding of the steroid to cytosol receptors; (b) a temperature dependent 'activation' of the receptor-steroid complex into a form capable of binding to nuclei; and (c) binding of the activated complex to nuclei modulating gene expression. The presence of a receptor protein for HC in embryonic chick retina cytosol has been demonstrated (9,10). In this communication, we report studies on the binding of the receptor-hydrocortisone (R-HC) complex with retina nuclei; these studies were done in a cell free system derived from 12-day embryonic chick retina cells. The purpose was to determine (a) the optimal conditions and specificity of this interaction, and (b) the number of nuclear acceptor sites for the R-HC complex and their chemical nature.

MATERIALS AND METHODS

Chromatographically pure 1,2,6,7,-^{3}H-HC (>80Ci/mMole) and 2,4,6,7-

*Present Address: Dept. of Biology, Illinois Institute of Technology, Chicago, Illinois 60616

**Abbreviations: Hydrocortisone-HC; Receptor-Hydrocortisone complex-R-HC; Glutamine Synthetase-GS; Tricine-Magnesium-Calcium buffer - TMC.

^{3}H-estradiol (106Ci/mMole) were obtained from New England Nuclear Inc. Neural retinas, dissected aseptically from 12-day embryonic chicks, were washed in Tyrode's solution, suspended in TMC buffer (0.02M tricine, 0.002M $MgCl_2$, 0.001M $CaCl_2$, pH 7.6) and disrupted in a Kontes glass homogenizer (five strokes with loose and five with tight pestle). The homogenates were spun at 800 x g to obtain the crude nuclear pellet. Cytosol was prepared by spinning the supernatant at 140,000 x g for one hour. The crude nuclear pellet was suspended in 0.5M sucrose in TMC, layered over 0.5ml of 3M sucrose and 1.5ml of 2.2M sucrose (both in TMC), and centrifuged at 58,000 x g for 60 minutes. Nuclei collected at the interface between 2.2 and 3M sucrose were withdrawn with a pasteur pipette, washed once with 0.5M sucrose in TMC and used for experiments.

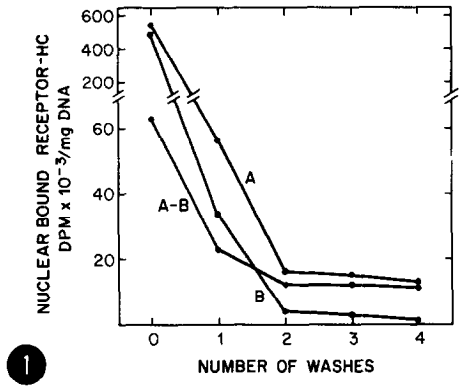
R-HC complexes were prepared by incubating retina cytosol preparations with a saturating dose (10) of ^{3}H-HC (5×10^{-8}M), in the presence or absence of 200 fold non-radioactive HC at 0°C for 1 hour. The total binding capacity of the cytosol was determined by charcoal assay (11,12).

Nuclear binding was measured by incubating triplicate samples of nuclei with cytosol preincubated with (a) ^{3}H-HC (5×10^{-8}M), referred to as A tubes, and (b) ^{3}H-HC (5×10^{-8}M) plus 200 fold non-radioactive HC, referred to as B tubes. After incubation, the nuclei were pelleted and washed twice with 0.5M sucrose in TMC. The final pellets were sonicated in 0.01M phosphate buffer, pH 7.1, and assayed for radioactivity, protein (13) or DNA (14). The difference in bound radioactivity between the A and the B tubes represented the amount of R-HC complex bound specifically. Figure 1 shows that after two washes this difference becomes constant, i.e. all non-specific counts are removed from the nuclei. This procedure was routinely used to determine specific nuclear binding.

RESULTS

Optimal conditions for nuclear binding: To determine the optimal conditions for nuclear binding, R-HC complexes prepared from retina cytosol were incubated with retina nuclei at different temperatures (0, 20, and 37°C); binding saturation occurred within 30 minutes at any of these temperatures with maximum binding at 20° (Fig 2). Separate experiments showed that the cytoplasmic receptor is heat-sensitive and its ability to complex with HC is almost completely lost during 15 minutes incubation at 37°C. Incubation of the cytosol alone at 20°C for one hour resulted in only 10% loss of this ability. Therefore, the reduced nuclear binding of the R-HC complex at 37°C is likely due to instability of the cytosol receptor, rather than to inability of the R-HC complex to bind to nuclei.

Cytosol receptors can be activated in two ways (5,15): (A) By



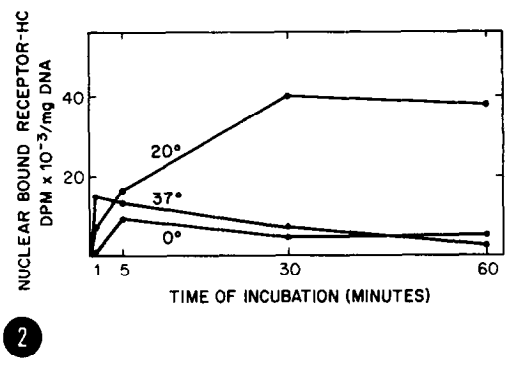
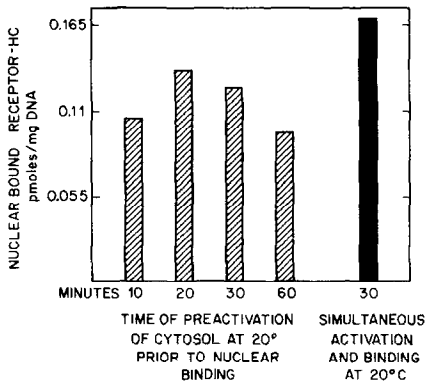


Fig. 1. Effect of washing on nuclear-bound R-HC complex. Retina cytosol (0.5ml) was incubated at 0°C for one hr with 5 x 10^{-8} M ^{3}H-HC in the absence (A tube) or presence (B tube) or 10^{-5} M non-radioactive HC. The mixture was then incubated with 0.4ml of nuclei (0.32 mg DNA) at 20°C for 30 min. After centrifugation, nuclear pellets were washed 0-4 times with 2 ml of TMC buffer containing 0.5M sucrose and assayed for radioactivity/mg DNA.

Fig. 2. Binding of R-HC complex to nuclei at different temperatures. Nuclei (0.12 mg DNA) were incubated at 0°, 20° or 37°C for the indicated times with cytosol containing the R-HC complex, in the presence or absence of competing non-radioactive steroid. Radioactivity/mg DNA was assayed in the washed pellets (see Methods).

preincubation of the R-HC complex at 20°C in the absence of nuclei; the activated complex can then bind to nuclei at 0°C. (B) By incubation of the R-HC complex together with nuclei at 20°C, in which case activation and nuclear binding proceed simultaneously. To compare the efficiency of these procedures, retina cytosol containing R-HC complex was incubated alone at 20°C for different times. This preactivated cytosol was added to nuclei; the mixture was incubated at 0°C for one hour and nuclear binding was then determined. This was compared with the amount bound when the same quantity of cytosol R-HC complex was directly mixed with nuclei and incubated at 20°C for 30 min. The results (Fig. 3) showed that preactivation (procedure A) was less efficient than activation in the presence of nuclei (procedure B). Data from this and several other experiments showed that even by procedure B a maximum of 50-60% of the R-HC complex could be activated, i.e. converted to a form capable of binding to nuclei (see Legend Figure 3).

Specificity of nuclear binding: Table I shows that the isolated retina nuclei retained binding specificity for R-HC complexes prepared from retina cytosol. Little or no binding occurred when nuclei were incubated in buffer containing only free tritiated HC. Similarly there was no significant binding to retina nuclei of estradiol-receptor complexes from retina cyto-



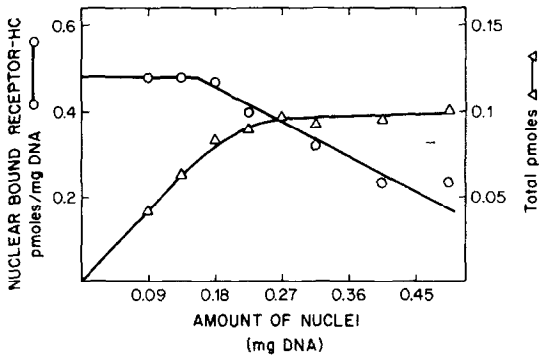


Fig. 3. Activation of the R-HC complex in the presence or absence of nuclei. Cytosol containing 0.11 pmoles of R-HC complex was (i) pre-activated at 20°C in the absence of nuclei for the indicated times (min), following which binding with nuclei (0.32mg DNA) was measured after one hr incubation at 0°C (hatched bars); (ii) directly mixed with nuclei and the mixture incubated at 20°C for 30 min for simultaneous activation and binding (solid bar). Out of the total of 0.11 pmoles R-HC complex in the cytosol, a maximum of 0.054 (0.17 pmoles/mg DNA x 0.32 mg DNA) was bound to nuclei, indicating a 50% activation.

Fig. 4. Binding of a constant amount of R-HC complex by increasing amount of nuclei. Aliquots of cytosol (0.5 ml containing 0.19 pmoles of tritiated R-HC complex) were incubated with the indicated amounts of nuclei at 20°C for 30 min in a total volume of 0.9 ml adjusted with TMC buffer. The nuclei were centrifuged, washed twice and assayed for amounts of radioactivity and DNA. A maximum of 0.1 pmoles of the complex was bound to nuclei indicating a 50-60% activation.

sol and R-HC complexes from embryonic mouse brain cytosol. HC complexed with proteins from serum of 20-day chick embryos also did not bind to the retina nuclei.

Total number of nuclear acceptor sites: The specificity of nuclear binding implied a finite number of acceptor sites for the R-HC complexes in the nuclei. Accordingly, exposure of increasing number of nuclei to a constant amount of R-HC complex should result, first, in saturation of all nuclear binding sites, followed by a decline of binding per nucleus. This expectation was borne out (Fig. 4). At low concentrations of nuclei, the amount of complex bound remained constant (approximately 0.5 pmoles per mg DNA). This value declined at higher concentrations of nuclei when the amount of R-HC complex became limiting with respect to the total number of nuclear acceptor sites. The total amount of R-HC complex bound to nuclei increased initially and then plateaued when all the acceptor sites were filled up (Fig. 4). As noted before, only 50-60% of the total available R-HC complex in the cytosol was activated and capable of binding to nuclei.

The amount of DNA per retina nucleus was determined to be 5 $\pm$ 0.5 pgms.

TABLE I

BINDING OF DIFFERENT RECEPTOR HORMONE COMPLEXES BY ISOLATED RETINA NUCLEI

Materials incubated with retina nuclei	Nuclear binding (pmoles/mg DNA)
(a) Buffer and ^{3}H-HC	0.02-0.04
(b) Retina cytosol and ^{3}H-HC (0.188 pmoles)	0.35
(c) Retina cytosol and ^{3}H-estradiol (0.03 pmoles)	0
(d) Mouse brain cytosol and ^{3}H-HC (0.13 pmoles)	0.004-0.008
(e) Chick serum and ^{3}H-HC (1.11 pmoles)	0.001

Buffer, cytosol or serum was incubated with tritiated hormones (5×10^{-8}M) in the presence and absence of excess non-radioactive hormone in a total volume of 0.5ml at 0°C for one hr to form the receptor-hormone complex; the mixture was then incubated with nuclei (0.14 to 0.23 mg DNA) at 20°C for 30 min. Nuclear binding was measured as described in Methods. The figures in parenthesis represent total amounts of receptor-hormone complexes exposed to nuclei, as determined by charcoal assay after the initial incubation. Using this value and the fact that nuclei bind, at saturation, 0.5 pmoles of R-HC complex per mg DNA, we estimate the number of acceptor sites for this hormone-receptor complex to be about 1500 per nucleus.

Effect of treatment of nuclei with enzymes on the acceptor sites: In preliminary tests on the chemical nature of the nuclear acceptor sites, nuclei were treated with DNAse or RNAse prior to incubation with cytosol R-HC complex. Table II shows that while treatment of nuclei with RNAse had no significant effect on R-HC binding, treatment with DNAse resulted in loss of approximately 70% binding ability. However this loss was not proportional to the amount of DNA degraded. Since the cytosol R-HC complex is resistant to DNAse, its reduced binding in the DNAse treated nuclei is not due to destruction of the complex by the enzyme. These data suggest that DNA may constitute the major component of the R-HC binding site. However, the fact that DNAse treatment reduces at most only 70% of the nuclear binding activity raises the possibility that nuclear proteins may also be involved in this binding.

DISCUSSION

The results presented here indicate that in a cell-free test system the binding characteristics of R-HC complexes to nuclei of embryonic neural retina cells are fundamentally similar to those reported for other steroids in several adult cell systems (5-7). This applies to the requirement for the initial complex formation of the steroid with the cytosol recep-

TABLE II

EFFECT OF ENZYMATIC DIGESTION OF NUCLEI ON THE BINDING OF RECEPTOR-HC COMPLEX

Treatment of the Nuclei	Nuclear-bound Receptor-HC (DPM/mg Protein)	Binding Activity lost (% of control)	Amount of DNA released (% of control)
None (control)	19495	0	0
DNase, 100µg/ml 0°, 60 minutes	6294	68	30
DNase, 100µg/ml 20°, 30 minutes	4887	75	75
Panc. RNase, 50µg/ml plus T1 RNase, 2µg/ml 0°, 60 minutes	22136	0	--

Suspensions of nuclei in TMC buffer with 0.5 M sucrose were treated with DNAse or RNAse as indicated. After treatment, nuclei were centrifuged, washed, resuspended in TMC buffer and incubated at 20°C for 30 min with cytosol containing the R-HC complex in the presence and absence of competing steroid. The amount of DNA released in the supernatants of the DNAse treated nuclei was assayed. Nuclear binding of R-HC complex was measured as described in Methods.

tor, to the temperature dependent activation of the R-HC complex and to the specificity of nuclear binding of this complex.

Assuming monovalency of binding sites, the total number of nuclear acceptor sites for the R-HC complex, in the presence of saturating amounts of the complex, was estimated to be about 1500 per nucleus; this estimate, derived from studies in a cell free system corresponds very well with that found for nuclei of intact retina cells (16). Studies on the nuclear binding of other hormone-receptor complexes showed that in rat hepatoma or in uterine cells there are approximately 8000 binding sites per diploid genome for receptor-dexamethasome and receptor-estradiol complexes respectively (17). Similarly, nuclei from rabbit fetal lung cells appear to have about 9000 binding sites for receptor-dexamethasone complexes per nucleus (18).

The relatively low number of nuclear acceptor sites for the R-HC complex in the embryonic neural retina cells could conceivably be due to the

involvement of only a portion of the cell population in the hormonal response; however, the available evidence does not support this assumption. Thus, the presence of only about 1500 R-HC binding sites per nucleus should facilitate further analysis of the relationship of hormone-genome interaction to GS induction and to other specific gene expressions in these cells.

ACKNOWLEDGEMENTS

This work is part of project supported by the following grants from the National Institutes of Health: HD01253 (A.A.M.), T01-HD 00297 (P.K.S.), HD-07110 (to the Biomedical Center for Population Research, University of Chicago) and 1-P01-CA 14599 (to the Cancer Research Center of the University of Chicago). We thank Dr. D.E. Koehler for advice and Barbara Schulak and Keith Balter for technical assistance.

REFERENCES

1. Moscona, A.A. in: Biochemistry of Cell Differentiation (A. Monroy ed.) Academic Press, London, 1971, p1.
2. Moscona, A.A., Moscona, M. and Saenz, N., Proc. Nat. Acad. Sci. U.S., 61, 160, 1968.
3. Sarkar, P.K. and Moscona, A.A., Proc. Nat. Acad. Sci. U.S. 68, 2308, 1971.
4. Sarkar, P.K. and Moscona, A.A., Proc. Nat. Acad. Sci. U.S. 70, 1667, 1973.
5. Jensen, E.V., Numata, M., Brecher, P.I. and DeSombre, E.R. in: Biochem. Soc. Symposium No 32 (R.M.S. Smellie ed.) Academic Press, London, 1971, p 133.
6. O'Malley, B.W., McGuire, W.L., Koehler, P.O. and Korenman, S.G., Recent Progress in Hormone Research, 25, 105, 1969.
7. Gorski, J., Shyamala, G. and Toft, T. in: Current Topics in Developmental Biology (A.A. Moscona and A. Monroy eds), Academic Press, New York, 1969, p149.
8. Mohla, S., DeSombre, E.R. and Jensen, E.V., Biochem. Biophys. Res. Comm., 46, 661, 1972.
9. Chader, G.J., Meltzer, R. and Silver, J., Biochem. Biophys. Res. Comm., 46, 2026, 1972.
10. Koehler, D.E. and Moscona, A.A., Manuscript in Preparation.
11. Korenman, S.G., Perrin, L.E. and McCallum, T.P., J. Clin. Endocrin. 29, 879, 1969.
12. Rousseau, G.G., Baxter, J.D. and Tomkins, G.M., J. Molec. Biol. 67, 99, 1972.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem., 193, 265, 1951.
14. Burton, K., Biochem. J. 62, 315, 1956.
15. Higgins, S.J., Rousseau, G.G., Baxter, J.D. and Tomkins, G.M., J. Biol. Chem., 248, 5866, 1973.
16. Sarkar, P.K. and Moscona, A.A., To be published.
17. Higgins, S.J., Rousseau, G.G., Baxter, J.D. and Tomkins, G.M., J. Biol. Chem., 248, 5873, 1973.
18. Ballard, P.L. and Ballard, R.A., Proc. Nat. Acad. Sci. U.S., 69, 2668, 1972.