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GLUCOCORTICOID HORMONES ARE SUCCESSIVELY PRESENT IN TWO SITES WITH DIFFERENT ACCESSIBILITIES TO NUCLEASES IN CHROMTIN FROM HTC CELLS

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Summary : Glucocorticoid hormones induce tyrosine aminotransferase synthesis in HTC cells after a lag of approximately 2 h. The question arises whether this lag corresponds to chromatin modifications related to the binding of the hormone. We have analyzed the accessibility to DNases of the hormone binding sites in chromatin from HTC cells incubated for various times in the presence of the hormone. After short incubations the hormone-receptor binding sites in chromatin are accessible to DNases, after longer incubations they become resistant to DNase digestion. The kinetics of the release *in vivo* of the hormone-receptor complex in an hormone free medium suggests the existence of 2 discrete classes of hormone binding sites in chromatin.

Steroid hormones act by binding to a specific receptor protein and subsequent interaction of the complex with chromatin acceptor sites. In hepatoma tissue cultured (HTC) cells, glucocorticoids induce the synthesis of tyrosine aminotransferase (TAT). A lag of approximately 2 h occurs between the addition of the hormone to the medium and the increase in TAT synthesis (1, 2). It is then interesting to determine whether some events at the chromatin level could be implicated. We have observed that glucocorticoid complexes associated with chromatin subunits can be easily released from the nucleus after a short nuclease digestion (unpublished date). Similar observations have been made in other systems (3-6). In this work we show that the hormone-receptor complex is successively associated with nuclease accessible sites and afterwards with resistant sites.

MATERIALS AND METHODS

Cell culture and hormone binding : HTC cells were grown in suspension in Swim's 77 medium supplemented with 10 % newborn calf serum as described by

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Hershko and Tomkins (7). For steroid treatment, the cells were cultured at 5.10^5 cells per ml, 5.10^{-8} M [^{3}H] Triamcinolone (Radiochemical Center - Amersham - Specific activity 29 Ci/mmole) were present in the medium for various times. TAT specific activity was measured according to Diamondstone (8) modified by Granner and Tomkins (9). Afterwards the cells were centrifuged at 600 g and the preparations were carried out at 0°C to avoid any dissociation of the complexes. The cells were washed as described by Pfahl et al (10), leading to less than 5 % of non specific binding. For nuclei preparation the cell membranes were disrupted by 0.2 % nonidet P_{40} as previously described (11).

Nuclease digestion : For DNase I digestion, the nuclei were washed and suspended in 10 mM Tris (pH 7.35), 10 mM NaCl, 3 mM $MgCl_2$ at a DNA concentration of 0.8-1.5 mg/ml (12). The nuclei were preincubated for 2 min at 37°C in the absence of the enzyme and equilibrated DNase I (Worthington) at 5-50 γ/ml was added for another 2 min at 37°C. The reaction was stopped in ice by addition of EDTA to a final 2 mM concentration. Undigested chromatin was removed by centrifugation at 1,500 g for 15 min. In all these experiments the material rendered acid soluble and the total released material were measured. Aliquots without DNase were used as controls. In that case 25-35 % of the radioactivity was spontaneously released from the nuclei, essentially in a free form. Corrections were made and the results expressed as percent of complexes released from chromatin by the DNase.

For micrococcal nuclease digestion the nuclei were washed in 0.3 M sucrose, 10 mM Tris (pH 7.3), 3 mM Ca^{2+} and suspended at a DNA concentration of 0.8-1.5 mg/ml in the same buffer except that Ca^{2+} was 1 mM (13). The incubations were performed under the same conditions as for DNase I, in the presence of 25 to 500 U/ml of micrococcal nuclease (Worthington).

Sucrose gradient centrifugation : After DNase digestion the supernatant was loaded on a 5-20 % linear sucrose gradient in 50 mM Tris (pH 8), 2 mM EDTA and centrifuged for 17 h at 39 000 rev/min in a SW 41 rotor at 3°C. The absorbance was automatically recorded and the radioactivity measured in each collected fraction.

Kinetics of in vivo dissociation : The cells were incubated for 45 min or 16 h in the presence of 5.10^{-8} M [^{3}H] Triamcinolone. The cells were then transferred in an hormone free medium and incubated for various times from 30 min to 6 h at 37°C under conditions described by Thompson et al (1) for the deinduction of TAT. The nuclei were then prepared as described above and the remaining radioactivity measured.

RESULTS

HTC cells were incubated for 45 min and for 16 h in the presence of [^{3}H] Triamcinolone. Nuclei were prepared and chromatin digested with various DNase I concentrations. The concentration of nuclear steroid receptor complexes was not significantly affected by the time of incubation of the cell in the presence of the hormone. It ranged from 40 000 to 50 000 sites per nucleus. Control experiments showed that in the absence of

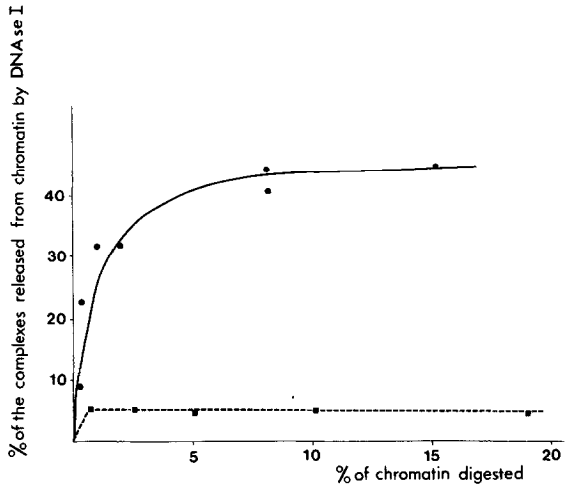

Figure 1. Release of glucocorticoid-receptor complexes by DNase I digestion. HTC cells were incubated for 45 min (●—●) and for 16 h (■- -■) in the presence of [^{3}H] Triamcinolone. Nuclei were prepared and submitted to DNase I digestion for 2 min at 37°C with increasing amounts of DNase I from 5 to 50 µg of enzyme per ml. Corrections were made for the radioactivity released in the absence of the enzyme, approximately 25-35 % of the radioactivity specifically associated with the nuclei. The results expressed the percentage of complexes released from chromatin by DNase I.

nuclease the amount of complexes spontaneously released from nuclei was the same (25-35 % of the radioactivity initially present in the nuclei) whatever the time of incubation. Approximately 50 % of the chromatin bound complexes were released from cells incubated for 45 min in the presence of the hormone, when 10 % of the chromatin was digested by DNase I. Only 5-7 % of the bound complexes were released from chromatin (corresponding to 2-3 000 binding sites) from cells which have been incubated for 16 h in the presence of the hormone whatever the level of digestion (fig. 1). Digestion with micrococcal nuclease led to the same results except that a higher level of chromatin digestion was required (date not shown).

The equilibrium of cytoplasmic-nuclease translocation is rapidly reached so that after 30 min of incubation, all the nuclear sites are apparently saturated. However TAT induction occurs only 2 h after hormone addition to the culture medium and reaches a maximum after 6-8 h. The same TAT induction kinetics were observed under our experimental conditions.

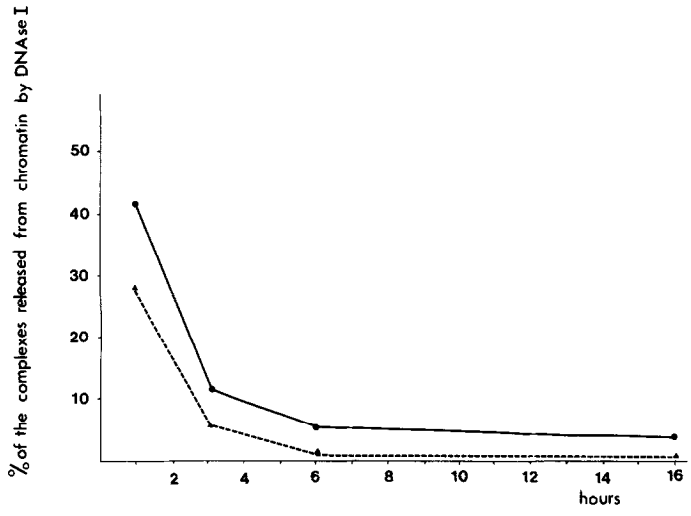

Figure 2. Effect of the time of incubation of the cells in the presence of the hormone on the release of the hormone-receptor complexes by DNase I. HTC cells were incubated for 45 min, 3 h, 6 h and 16 h in the presence of [^{3}H] Triamcinolone. Nuclei were prepared and submitted to digestion with increasing amounts of DNase I from 5 to 50 µg/ml. Corrections were made for the hormone spontaneously released as indicated in the legend to Fig 1. The results expressed the percentage of the complexes released when 1.5 % (▲- - -▲) and 5 % (●——●) of the chromatin were digested.

Fig. 2 shows that in cells incubated for 45 min in the presence of the hormone, almost 30 % of the hormone complexes were released by DNase I when 1.5 % of the chromatin was digested, whereas after 6 h of incubation more than 90 % of the chromatin binding sites were resistant to DNase I even when 5 % of the chromatin was digested. This change in the accessibility of the hormone binding sites does not reflect a general modification in the accessibility of chromatin to DNases since identical kinetics of digestion have been observed with chromatin from HTC cells not treated with the hormone, incubated for 45 min or incubated for 16 h in the presence of glucocorticoid.

Chromatin samples from HTC cells incubated for 45 min and for 16 h in the presence of [^{3}H] Triamcinolone were each digested with micrococcal nuclease under conditions which rendered 2.1 and 2.6 % of the DNA acid soluble respectively and the released material was analyzed by centrifugation on a 5-20 % linear sucrose gradient. The familiar pattern of

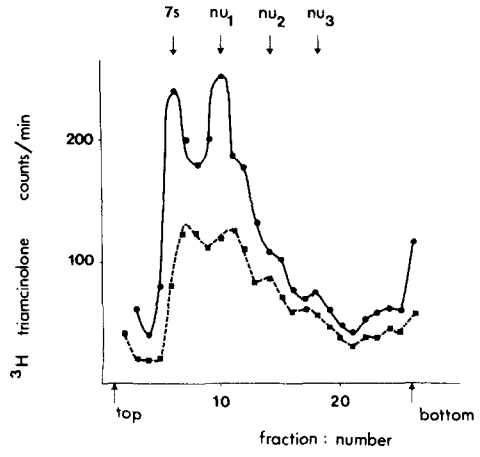

Figure 3. Sucrose gradient analysis of the chromatin fragments released by micrococcal nuclease digestion. HTC cells were incubated at 37°C for 45 min (●——●) and for 16 h (■- -■) in the presence of [^{3}H] Triamcinolone. The nuclei were submitted to digestion by 50 µg/ml of micrococcal nuclease for 2 min at 37°C. The released material was analyzed on a 5-20 % linear sucrose gradient.

chromatin fragments was observed (14, 15). The radioactive patterns (fig.3) obtained with both samples were not significantly different, except for the total amount of released material ; in both cases two major peaks, corresponding respectively to 7 S and to mononucleosomes, were obtained. The 7 S peak could correspond to the hormone-receptor complex associated with a small fragment of DNA. Some radioactivity was also found associated with dimeres and trimeres of nucleosomes.

When the hormone was removed from the medium, there was a rapid decrease in TAT activity and in the concentration of bound hormone (1). We have studied the decay of chromatin bound hormone in cells which have been incubated respectively for 45 min and for 16 h in the presence of the hormone. Fig. 4 shows that (a) the decay was much faster in chromatin from cells which have been incubated for 45 min, (b) in both cases there was a unique class of binding site.

DISCUSSION

These observations show that the time of incubation of the cell in the

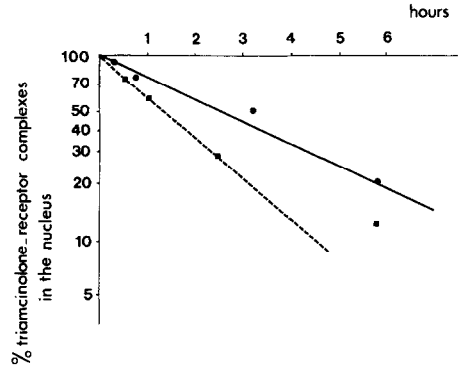

Figure 4. *In vivo* dissociation of hormone-receptor complexes. HTC cells were incubated with [^{3}H] Triamcinolone for 45 min (■---■) and for 16 h (●——●). The cells were then transferred in an hormone free medium and incubated at 37°C. At various times aliquots were collected, nuclei prepared and their radioactivity measured.

presence of the hormone strongly influences the manner by which the hormone-receptor complexes are associated with chromatin. Essentially 2 types of binding sites were found characterized by a different accessibility to nucleases. We do not know whether the hormone-receptor complexes move from one type of binding site to the other or if the hormone modifies the chromatin binding site in such a way that it becomes less accessible to DNases. From the kinetics of dissociation in an hormone free medium the nuclease hormone-receptor complexes appear stabilized by a previous long incubation of the cells in the presence of the hormone. Since most of the complexes were dissociated at the same rate in cells preincubated in the presence of the hormone for a given time, all the nuclease sites appear equivalent in these cells. Nevertheless we cannot exclude the presence of a small number of unstable complexes.

A modification in chromatin structure shortly after the addition of hormone has been described in various systems. In HTC cells steroids do not appear to affect the accessibility of total chromatin to DNases, nevertheless a limited number of specific regions could have been modified.

It is tempting, even if no demonstration is given by our results, to

assume that the hormone induced synthesis of TAT occurs only when the hormone is present in the less accessible class of binding sites. A better knowledge of the fine structure of the chromatin region including the acceptor sites of the hormone-receptor complexes and its spatial relationship with the structural gene of TAT is required for the understanding of the molecular mechanism of hormone regulation of enzyme synthesis.

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References

1. Thompson, B.E., Tomkins, G.M. and Curran, J.F. (1966) Proc. Natl. Acad. Sci. USA 56, 296-303
2. Baxter, J.D., and Tomkins, G.M. (1970) Proc. Natl. Acad. Sci. USA 65, 709-715
3. Massol, N., Lebeau, M.C. and Baulieu, E.E. (1978) Nucleic Acids Res. 5, 723-738
4. Senior, M.B. and Frankel, F.R. (1978) Cell 14, 857-863
5. Rennie, P.S. (1979) J. Biol. Chem. 254, 3947-3952
6. Cidlowski, J.A. and Munck, A. (1980) Biochim. Biophys. Acta 630, 375-385
7. Hershko, A. and Tomkins, G.M. (1971) J. Biol. Chem. 246, 710-714
8. Diamondstone, T.J. (1976) Analyt. Biochem. 16, 395-401
9. Granner, D.K. and Tomkins, G.M. (1970) Meth. Enzym. 17, 633-637
10. Pfahl, M., Sandros, T. and Bourgeois, S. (1978) Molecular and Cellular Endocrinology 10, 175-191
11. Defer, N., Crepin, M., Terrioux, C., Kruh, J. and Gros, F. (1979) Nucleic Acids Res. 6, 953-966
12. Weintraub, H. and Groudine, M. (1976) Science, 193, 848-853
13. Shaw, B.R., Corde, J.L., Sahasrabuddhe, C.G. and Van Holde, K.E. (1974) Biochem. Biophys. Res. Commun. 61, 1193-1198
14. Noll, M. (1974) Nature 251, 249-251
15. Kitzis, A., Tichonicky, L., Defer, N. and Kruh, J. (1980) Eur. J. Biochem. In press.