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NUCLEAR ACCEPTOR SITES FOR GLUCOCORTICOID RECEPTORS

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SUMMARY

Glucocorticoids increase the rate of synthesis of tyrosine transaminase in hepatoma tissue culture cells. The first steps in this hormonal action involve specific binding of steroid to a cytoplasmic receptor followed by interaction of the complex with the nucleus.

To investigate the nature of nuclear acceptor sites, a cell-free system was designed in which nuclei isolated from hepatoma cells bind specifically the receptor–steroid complex. DNA appears to be involved in this process. Since binding of receptors to isolated nuclei resembles in many ways the corresponding interaction taking place in the intact cell, binding of receptors to pure DNA was studied in greater detail. Contrary to what is seen with whole nuclei, there is no evidence that DNA contains a limited number of sites for glucocorticoid receptors. It is concluded that DNA may be a necessary but not sufficient component of the chromatin acceptor sites.

INTRODUCTION

Induction of tyrosine aminotransferase (TAT) by steroids in rat hepatoma tissue culture (HTC) cells is being studied as a model of glucocorticoid hormone action[1]. HTC cells contain specific proteins, called receptors, that bind these hormones with high affinity [2, 3] and are considered to be mediators of the hormonal action[2, 4].

After fractionation of cells grown in absence of steroid. receptors are found exclusively in the cytosol[3]. However, when intact cells have been exposed to a glucocorticoid like dexamethasone or cortisol, most receptors become associated with the nucleus[2, 5]. The physiological importance of cytosol receptor binding to nucleus is buttressed by the fact that this interaction is differently influenced by steroids endowed with a different biological activity[2, 5]. This paper summarizes our current knowledge concerning the number and chemical nature of the nuclear acceptor sites in the HTC system.

RESULTS AND DISCUSSION

A. *Binding of receptor–glucocorticoid complex to isolated nuclei*

One way of determining whether there is a homogeneous and finite population of nuclear sites is to study the equilibrium kinetics of the nuclear binding reaction over a wide range of receptor–steroid complex concentrations. This was achieved by exposing isolated nuclei to cytosol containing receptor labelled with radioactive dexamethasone. It was found that the nuclear binding reaction results from three distinct steps: (1) formation of a complex between cytosol receptor and steroid; (2) activation of the complex; (3) binding of the complex of the nucleus[6, 7]. The first step occurs readily at 0° and at low salt concentration. Activation of the complex can be achieved by raising the temperature or the ionic strength. There is evidence this step is a distinct molecular event that also takes place in the intact cell[5]. The cell-free nuclear binding reaction can itself occur at 0° when using activated receptor–steroid complex. Figure 1 shows the results of an experiment in which HTC nuclei were exposed to increasing concentrations of activated cytosol containing receptors saturated with [^{3}H]-dexamethasone. Competitive inhibition of binding of radioactive complex to nucleus was observed when

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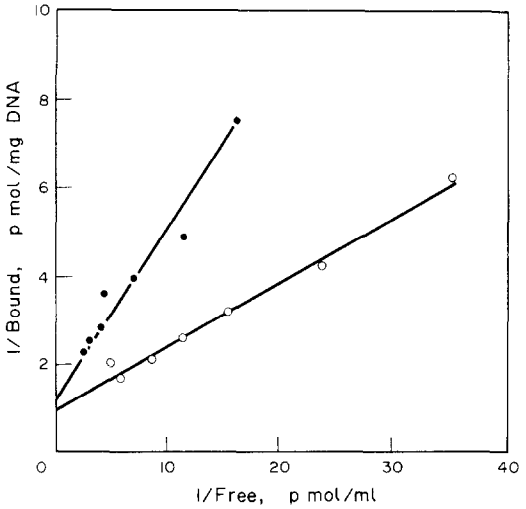

Fig. 1. Saturable binding of [^{3}H]-dexamethasone–receptor complex to isolated nuclei: competitive inhibition by receptor bound with nonradioactive dexamethasone. HTC nuclei were incubated at 0° with increasing amounts of activated HTC cytosol saturated with [^{3}H]-dexamethasone in the absence (○) and presence (●) of a constant amount of competing HTC receptor bound with nonradioactive dexamethasone (redrawn from Ref. [7]).

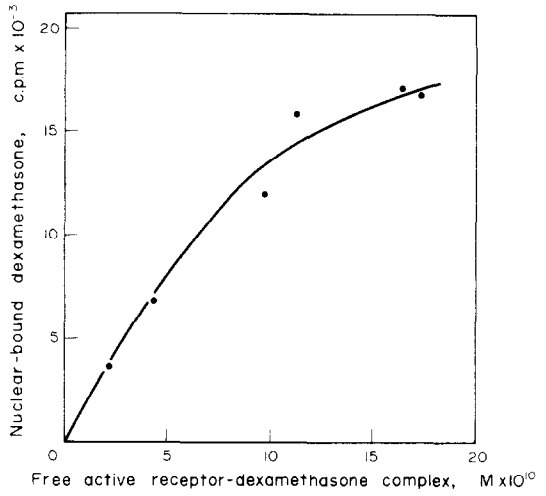

Fig. 2. Binding of glucocorticoid receptors to isolated nuclei at constant cytosol concentration. Aliquots of HTC cytosol were preincubated with various concentrations of [^{3}H]-dexamethasone in order to vary the extent of saturation of cytosol receptor with steroid. After activation with 0·15 M NaCl at 20° for 30 min, these cytosol aliquots (0·7 ml) were incubated at 0° with HTC nuclei. The concentrations of active receptor–steroid complexes and nuclear-bound receptors were determined as described in Ref. [7]. Half-maximum binding is obtained at a concentration of free complex higher than in Fig. 1 because the incubation contained 0·15 M NaCl which decreases the apparent affinity of nuclear binding[7].

adding a constant amount of activated HTC cytosol containing receptors complexed with nonradioactive dexamethasone. The linear relationship and common intercept on the ordinate are compatible with high affinity (K_d 0° $= 2 \times 10^{-10}$ M) reversible binding of the complex to a homogeneous and limited population of sites (1·6 pmole/mg DNA) in the nucleus[7].

It is possible that cytosol factors other than the receptor interfere with binding of the complex to the nucleus[7]. Since pure receptor is not currently available an experiment was designed in which the only variable was the concentration of cytosol receptor (Fig. 2). Nuclei were incubated in the presence of constant amounts of activated cytosol in which concentration of the complex was varied by using subsaturing concentrations of [^{3}H]-dexamethasone. Under these conditions, nuclear binding retains the characteristics described in Fig. 1 suggesting that saturability of the process is really due to occupancy of a finite number of acceptor molecules.

Further investigation[7, 8] provided experimental evidence that binding of activated receptor–steroid complex to isolated nuclei resembles the interaction of receptor with nucleus in the intact cell[5]:

(1) in both systems free receptor does not bind to nucleus unless it is complexed with a glucocorticoid;

(2) activation of the complex is required;

(3) complexes bound to nucleus in the intact cell and in cell-free experiments dissociate from the nucleus at similar rates;

(4) in both cases the sensitivity of the complex to extraction from nucleus by NaCl is the same;

(5) complexes formed in the intact cell or in isolated nuclei and subsequently released by NaCl have the same sedimentation velocity in glycerol gradients;

(6) most important, the number of acceptor sites per isolated nucleus (about 15,000 assuming one acceptor site per steroid molecules) is very similar to the maximum number (about 13,000) of steroid molecules specifically bound per nucleus in the intact cell;

(7) in both systems nuclear binding requires the integrity of DNA; this is developed below.

B. *Binding of glucocorticoid receptor to DNA*

Treatment of isolated nuclei with DNase abolishes their capacity to bind receptor–steroid complex[6]. This is not due to the nonspecific destruction of nuclear architecture. First, when the enzyme is added only after receptor has interacted with nuclei the latter do not release bound complexes[6]. Second, DNase-treated nuclei retain their ability to bind other types of steroid receptors[9]. It is concluded that DNA may come into play for binding of glucocorticoid receptors to isolated nuclei. Therefore, if the cell-free system is an adequate model, it is possible DNA is

Table 1. Effect of BudR on receptor binding to the nucleus of intact HTC cells

Precursor	Nuclear-bound dexamethasone pmole/mg DNA	% left after sucrose	% left after NaCl
TdR	1·70	93	29
BudR	1·77	97	53

HTC cells, grown for 3 days with 10^{-5} M thymidine (TdR) or bromodeoxyuridine (BudR) were exposed for 45 min to $2{\cdot}5 \times 10^{-8}$ M [^{3}H]-dexamethasone with or without competing nonradioactive dexamethasone. Specific nuclear binding was determined[5] before and after exposure of nuclei for 45 min to 0·25 M sucrose or 0·3 M NaCl. That radioactivity released from nuclei was still bound to receptor was verified by gel filtration.

also involved in nuclear binding of receptor in the intact cell. The following experiments suggest it is indeed the case. First, when nuclei isolated from cells incubated with [^{3}H]-dexamethasone are fractionated, most of the specifically-bound receptors are found in the chromatin (Higgins, S. J., unpublished). Second, *in vivo* chemical substitutions in the DNA molecule influence receptor binding to nucleus in the intact cell (Table 1). The salt-dependence of receptor release from nuclei bound in the whole cell was compared in cells grown in the presence of either thymidine of bromodeoxyuridine. In the latter, 50% of the thymidine residues in the DNA are replaced by bromodeoxyuracil. This treatment conferred to the nuclei a higher affinity for the receptor as witnessed by the increased resistance to elution of receptor by NaCl.

Thus, cell-free binding of cytosol receptor to DNA was studied to determine whether this interaction could account for receptor binding to isolated nuclei. Pure HTC cell DNA was incubated at 0° with increasing concentrations of activated cytosol labelled with [^{3}H]-steroid. DNA-bound receptor was then separated from free complexes by agarose gel filtration[6]. Detailed studies with this system[10] led to the following observations:

(1) receptor must be complexed with an active glucocorticoid before it can bind to DNA;

(2) receptor activation is not an absolute requirement for binding to DNA; however, activation increases the affinity of the complex for DNA;

(3) DNA's of mammalian, bacterial of phage origin all have the same binding affinity for receptor-steroid complex; the affinity of denaturated DNA is lower; that of RNA is negligible;

(4) there is no evidence DNA can be saturated with concentrations of receptor–steroid complex that are more than sufficient to saturate the acceptor sites in isolated nuclei; this was the case with both native and denaturated DNA as well as with partially purified receptor (Fig. 3).

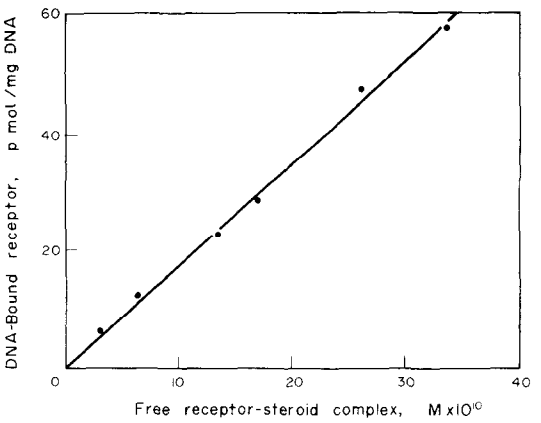

Fig. 3. Binding of glucocorticoid receptor to DNA. HTC cytosol bound with [^{3}H]-triamcinolone acetonide was partially purified as described elsewhere[10]. Portions (5–50 μl) of the receptor preparation were incubated at 0° with 10 μg of pure HTC DNA (final volume 0·3 ml) and DNA binding was measured by agarose gel filtration[6].

These results have to be reconciled with those obtained in isolated nuclei where acceptor sites can become saturated with receptor. If DNA is part of the physiological acceptor sites one has to conclude that its accessibility is restricted in chromatin, providing one relies upon information obtained with isolated nuclei. Another interpretation would be that isolated nuclei have lost some of the properties they enjoy in the intact cell and are inadequate for mimicking nuclear binding[8]. In any case, it appears unlikely that receptor binds directly to specific sequences in "open regions"[11] of the DNA without involvement of other chromatin components.

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DISCUSSION

Schrader:

I have a question about the BUDR experiments. When you grew the cells in BUDR did they continue to cycle?

Rousseau:

Yes, they were exposed for three days to 10^{-5} M BUDR or TDR and growth was unaffected (Stellwager and Tomkins, *J. Mol. biol.* **56** (1971) 167).

Schrader:

We had looked at the binding of receptors to DNA following a most interesting report a couple of years ago by Baxter *et al.* who thought that DNA binding saturated. We took a look at it and saw that it did saturate. I haven't gone back and done any more of that. I'm curious to know what happened to DNA saturation.

Rousseau:

The first experiments suggested that indeed there was apparent saturation of DNA with the complex. Those consisted of incubating a given amount of DNA with increasing amounts of cytosol saturated in terms of receptor-steroid complex. Since other cytosol proteins did bind to DNA, it was important to repeat the experiment using a constant amount of cytosol and varying only the free steroid concentration in order to produce various extents of saturation of the receptor. Secondly, the cytosol is not devoid of deoxy-ribonuclease activity. In our technique the mixture of DNA and receptor complex is filtered over agarose. It was assumed that the amount of DNA which came out from the agarose column was the same as the amount loaded. This was the case. In the experiments we report now, the actual amount of DNA in the eluate is measured and the results are expressed as amount of receptor bound per actual amount of DNA present. Under those conditions and taking care of the DNAase activity, we do not get any linear saturation any more.

Vorob'ev:

Have you got any evidence that there is a difference between the binding constant of the receptor hormone complexes with DNA and chromatin or nuclei?

Rousseau:

Since there is no saturability of DNA, we cannot attribute a binding constant to this interaction because it looks as if this is a binding of low affinity to an infinite number of sites. As far as the isolated nuclei are concerned, we consistently find an apparent dissociation equilibrium constant at 0°C of about 2×10^{-10} M receptor-steroid complex. This is not quite clear in the case of nuclei in the intact cell. Here the apparent limited nuclear capacity could merely be due to the limited availability of cytosol receptors. Experiments are in progress to answer this question.

Vorob'ev:

Chromatin isolated from target cells and other types of cells differs in the ability to bind receptors. You can also see some physical-chemical differences between these chromatins. I don't think that your data proves that DNA itself is involved in the hormone–receptor binding. Maybe this is only a specific conformation of chromatin in nuclei that determines the organization of acceptor sites for the binding of hormone–receptor complexes.

Rousseau:

Yes, I agree that this is quite an indirect argument but at least it indicates that if anything happens to chromatin in the intact cell, nuclear binding is affected. Therefore, the receptor is likely to bind to sites that are close to DNA rather than to nuclear membrane or to nucleoli.

Gurpide:

Have you noticed any heterogeneity in the type of steroid binding complexes that you extract from the nuclei?

Rousseau:

It looks as if the nuclear acceptor sites all belong to a homogeneous population in terms of the kinetic data we do have. In fact there doesn't seem to be any non-specific binding of the complex to the nucleus as we see with free steroid for instance. By Scatchard analysis the acceptors appear to belong to a homogeneous population.

Munck:

Can you estimate the capacity of the DNA to bind the hormone–receptor complex at a concentration of hormone–receptor complex that might be expected in the cell? How does that compare to the capacity of the nuclei? Also, have you measured as a function of salt concentration the degree of activation of the inactive hormone–receptor complex?

Rousseau:

I will first answer the last question concerning the salt-dependence for activation being measured in this instance as the ability of the complex to bind to the nuclei. At 0°C we

get maximum activation at 0·3 M NaCl but routinely we activate by incubating the complex at 20° for half an hour in 0·15 M NaCl which is equivalent to what we obtain at 0·3 M NaCl. We then filter over G25 Sephadex to remove the excess salt. Concerning the first question, the extent of DNA binding at complex concentrations one would expect in the intact cell is much higher than with isolated nuclei.