Glucocorticoid Receptor Binding to Calf Thymus DNA. 1. Identification and Characterization of a Macromolecular Factor Involved in Receptor–Steroid Complex Binding to DNA

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ABSTRACT: Activation of receptor–steroid complexes to a form with high affinity for DNA is a poorly understood process involving multiple components in addition to the holoreceptor. Employing rat HTC cells as the source of glucocorticoid receptor, we show that maximal receptor binding to calf thymus DNA is mediated by a previously unknown small molecular weight factor. This factor can be removed from cytosolic preparations of receptor by gel filtration chromatography. Salt extraction of crude nuclear pellets afforded much larger amounts of a similar DNA-binding activity factor. The cytoplasmic factor and the more abundant nuclear factor were identical on the basis of their similar physical properties. The factor was precipitable in the crude state with $(NH_4)_2SO_4$ and stable to heat as well as freezing and thawing. Chromatography on DNA–cellulose revealed that the factor itself did not bind to DNA. The factor could be filtered through a Centricon C-3 microconcentrator (molecular weight cutoff ~3000) but was excluded from Sephadex G-10 columns. These parameters enable us to determine an apparent molecular weight of 700–3000 for this factor. The presence of large amounts of this factor in nuclei accounts for the previously unexplained observation that, following size exclusion chromatography, more activated complexes bind to nuclei than to DNA. These data indicate that some, but not all, of the activated complexes require factor to be able to bind to DNA. The predominantly nuclear localization of this factor, coupled with its ability to increase DNA binding, attests to the biological relevance of this factor in the whole cell action of receptor–glucocorticoid complexes.

Steroid-induced modulation of gene transcription in whole cells occurs only after the completion of a specific sequence of receptor-mediated events. The steroid first diffuses through the cell membrane and binds to a receptor protein which, at least for glucocorticoid receptors, is predominantly cytoplasmic (Antakly & Eisen, 1984; Gustafsson et al., 1987). The receptor–steroid complex then undergoes a process termed activation whereby the affinity of complexes for DNA and nuclei is dramatically increased (Baxter et al., 1972; Kalimi et al., 1975; Milgrom, 1981). Once activated, the complexes bind to specific DNA sequences (Payvar et al., 1983; Beato et al., 1987) in the nucleus and, in an unknown manner, influence the transcription of specific genes.

A more detailed understanding of receptor action has been complicated by the large number of nonreceptor components that have been implicated in receptor–steroid action [see reviews by Simons (1979), Schmidt and Litwack (1982), Housley et al. (1984), and Isohashi and Sakamoto (1986)]. Thioredoxin keeps the whole cell receptor in a reduced form that binds steroid (Grippo et al., 1986). Several proteins are thought to be associated with the unactivated receptor–steroid complex. Among these are an ~59K protein (Tai et al., 1986; Rexin et al., 1988) as well as hsp90 (Bresnick et al., 1989). A low molecular weight inhibitor of activation (Bodine & Litwack, 1988a,b; Meshinchi et al., 1988), and possibly RNA (Webb et al., 1986; Sablonniere et al., 1988), may also be associated with the unactivated complex. Additional components have been found to influence the action of activated complexes (Simons et al., 1976; Schmidt & Litwack, 1982; Dahmer et al., 1985; Isohashi & Sakamoto, 1986; Tymoczko et al., 1988; Cavanaugh & Simons, 1990). Furthermore, activated complexes may selectively bind to certain tRNAs (Ali & Vedeckis, 1987).

Considerable attention has recently been focused on the process of activation and the binding of activated complexes to DNA. Numerous experimental conditions have been described which stimulate activation in vitro, e.g., dialysis (Sato et al., 1980), dilution, heating, increased salt concentration or pH, $(NH_4)_2SO_4$ precipitation, RNase, and ATP (Milgrom, 1981). While the subsequent binding of activated complexes to those biologically active DNA sequences known as steroid regulatory elements (SREs)[1] is undeniably crucial for steroid-mediated gene transcription, the importance of binding to nonspecific DNA sequences should not be overlooked. Nonspecific DNA facilitates the binding of trans-acting factors to specific sequences (Berg et al., 1982). Furthermore, without nonspecific DNA binding to act as a buffer, activated complex binding to a small number of high-affinity SREs would become saturated, and would cause full induction or repression of responsive genes, over a very narrow concentration of steroid (Winter & von Hipple, 1981). Partially for these reasons, and due to the ready availability and ease of experimental manipulations, nonspecific DNA (particularly DNA–cellulose) has been used in most studies of activation. However, it has

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[1] Abbreviations: DCC, dextran-coated charcoal; Dex or dexamethasone, 9α-fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; EDTA, ethylenediaminetetraacetic acid; HAP, hydroxylapatite; HTC, hepatoma tissue culture; SRE, steroid regulatory element; TAPS, 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonic acid.

been previously observed by us (Simons, 1977) and others (Tienrungroj et al., 1987b) that, under some conditions, more activated receptor–steroid complexes bind to nuclei than to DNA. The initial purpose of the present study, therefore, was to resolve the differences between nuclear and DNA binding of activated glucocorticoid complexes. We now report that a low molecular weight component is required for the maximal binding of receptor–steroid complexes to DNA. This DNA-binding activity factor can be removed from cytosolic preparations of receptor by gel filtration chromatography. Further studies indicated that the cytosol contains two factors in a ratio of ~2:1 and that the nuclear pellet of lysed cells contains even more of a similar DNA-binding activity factor. We have compared the physical and biochemical properties of the cytosolic and nuclear pellet factors and conclude that the major cytosolic factor is the same as the more abundant nuclear pellet factor.

Experimental Procedures[2]

Materials. [1,2,4-^{3}H]Dexamethasone (Dex)[1] (40 Ci/mmol) was purchased from Amersham. Hepatoma tissue culture cells (HTC) were grown in Swim's S-77 media (Gibco) supplemented with 5% fetal bovine serum and 5% newborn calf serum (Bio–Fluids). Sigma supplied nonradioactive Dex and trypsin as well as Sephadex G-10 and G-50. Native calf thymus DNA–cellulose and Sephadex G-25 were purchased from Pharmacia. Crystalline TAPS was from Calbiochem-Behring, phosphate-buffered saline from Quality Biological, NaSCN from Aldrich, $(NH_4)_2SO_4$ from Schwarz/Mann, and NaCl from Baker. Hydroxylapatite (HAP) was purchased from Bio-Rad and DE-52 (DEAE-cellulose) from Whatman. Pronase, RNase A, and DNase I were all from Boehringer-Mannheim. Centricon C-10 and C-3 microconcentrators were purchased from Amicon Corp. The scintillation cocktail used was Hydrofluor, manufactured by National Diagnostics.

Preparation of Activated Receptor–Glucocorticoid Complexes. HTC cells were grown in spinner flasks as described elsewhere (Thompson, 1979). Crude cytosolic receptors were prepared by the freeze–thaw method of Reichman et al. (1984). Glucocorticoid receptors were bound with 5×10^{-8} M [^{3}H]Dex ± 500-fold excess [^{1}H]Dex for 2.5 h and total receptor–steroid complexes determined by dextran-coated charcoal (DCC), exactly as described by Simons and Miller (1984). Receptor–steroid complexes were activated by adding an equal volume of TAPS buffer (25 mM TAPS, 1 mM EDTA, 10% glycerol, pH 8.8 at 4 °C), adjusting the solution to 50 mM β-mercaptoethanol, and incubating at 20 °C for 30 min.

Preparation of Crude and Washed Nuclei. Nuclei were prepared from HTC cells grown to a density of $(4–6) \times 10^5$ cells/mL as previously described (Simons et al., 1976). Thus cells were washed free of media by three washes with phosphate-buffered saline and ruptured by freezing in liquid N_2. The thawed pellet, designated crude nuclei, was resuspended in TAPS buffer and vortexed vigorously. In experiments employing washed nuclei, the pellet resulting from the freeze–thaw step was washed twice with TAPS buffer. Microscopic examination revealed intact nuclei with few cytoplasmic tags.

Sephadex Gel Filtration Chromatography and Preparation of Factor-Depleted Activated Complexes. All chromatography resins were equilibrated in TAPS buffer according to manufacturers' instructions. Columns (5.0 cm × 1.5 cm) with a 9-mL bed volume were poured and washed with fresh TAPS buffer shortly before use. To prepare factor-depleted complexes, 500 μL of cytosol was loaded onto each column, which was developed with TAPS buffer. Aliquots (0.5 mL) were collected and counted to determine the peak fractions containing the [^{3}H]Dex-bound receptors. Routinely, the two highest dpm fractions were pooled in each experiment. In order to desalt samples, 100-μL aliquots were chromatographed on mini-Sephadex G-10 columns (0.55 mL of Sephadex in a 1-mL tuberculin syringe). The columns were washed with 100 μL of TAPS buffer, and the excluded volume was obtained with an additional 100 μL of TAPS buffer.

Preparation of Cytosolic and Nuclear Extract Factors. Cytosol solutions of factor were prepared by incubating HTC cytosol with 2.5×10^{-5} M [^{1}H]Dex. For nuclear extract factor, the low-speed (37000g) pellet of nuclei generated from 10^9 cells during preparation of HTC cytosol (Reichman et al., 1984) was vortexed vigorously with 6 mL of 0.5 M NaCl in TAPS buffer. The mixture was kept on ice for 1 h with frequent mixing. After centrifugation (3000g/10 min), the factor was precipitated with 40% saturated $(NH_4)_2SO_4$ for 1 h, pelleted (3000g/10 min), resuspended in 5 mL of TAPS buffer, aliquoted into 1-mL portions, and stored at −78 °C. All nuclear extracts were desalted on Sephadex G-10 columns before use.

Nuclear Binding Assay. In a modification of our previous assay (Simons, 1977) activated receptor–steroid complexes with [^{3}H]Dex ± [^{1}H]Dex were incubated in duplicate with $\sim 1.5 \times 10^7$ of either crude or washed nuclei for 2 h. The nuclei were centrifuged (Sorvall CW-1 centrifuge at high setting/1 min) and washed twice with 0.5 mL of TAPS buffer. [^{3}H]Dex-bound complexes were extracted for 30 min with 0.3 M NaSCN in TAPS bufffer with frequent mixing (Simons & Miller, 1986). After centrifugation (CW-1/1 min), the amount of nuclear-bound receptors (corrected for supernatant volume) was determined by subtracting the radioactivity bound in the presence of [^{1}H]Dex and is expressed as percent of added complexes.

Activated Complex Binding to DNA/DEAE or DNA/HAP Minicolumns. The percentage of receptor–steroid complex that could bind to DNA was quantitated by a modification of the minicolumn procedure of Holbrook et al. (1983) which used only two columns joined together with a piece of polyethylene. All resins were equilibrated in TAPS buffer. The top column was composed of 0.3 mL of native calf thymus DNA–cellulose while the bottom column contained 0.3 mL of either HAP or DEAE-cellulose. After the samples were loaded, the columns were washed with 7 mL of TAPS buffer under pressure. The resins were "dried" with 10 mL of air under pressure and transferred directly to separate scintillation vials for counting. Recovery of total complexes on both columns was always about 60–70% of the receptor content of unactivated cytosols, as determined by DCC. Control experiments employing double DNA columns indicated that a single 0.3-mL DNA column bound 96.9 ± 0.5% (SD, $n = 7$) of the complexes capable of binding to DNA.

Reconstitution Experiments. Routinely, 40 μL of cytosol which had been depleted of factor by Sephadex G-50 chromatography was mixed with the sample to be tested for factor activity. The volume of the mixture was brought to 120 μL with TAPS buffer and incubated for 20 min. The sample was then loaded onto a DNA/DEAE (or DNA/HAP) minicolumn, and the amount of activated complex bound to each resin was assayed as described above.

[2] Unless otherwise indicated, all manipulations were performed at 0 °C.

Table I: Binding of Activated Glucocorticoid Complexes to Nuclei and DNA

binder	% binding of glucocorticoid complexes in cytosol	cytosol through Sephadex G-25
	Part A[a]	
crude nuclei	45	55
washed nuclei	46	62
DNA	49	30
	Part B[b]	
DNA	81	35

[a] Activated cytosol (100 μL) containing 75600 dpm of [^{3}H]Dex-bound receptors (determined before activation by DCC) were incubated with 100 μL of crude or washed nuclei. Nuclear-bound receptor–steroid complexes were quantitated as described under Experimental Procedures. An aliquot of activated cytosol (50 μL) was also assayed on DNA/HAP minicolumns. The same activated cytosol was also chromatographed on a Sephadex G-25 column, and aliquots (100 μL containing 24066 dpm) were assayed for binding to nuclei or DNA/HAP minicolumns. All data are expressed as (dpm of DNA or nuclear-bound complexes/dpm of added complexes) × 100. [b] The data in (A) are presented in a different manner to show the relative amount of recovered complexes that bind to DNA. Recovery of complexes on minicolumns was 60% for activated cytosol and 85% for Sephadex G-25 chromatographed activated cytosol. The value shown in part B equals (dpm of complexes bound to DNA/dpm of complexes bound to DNA + HAP) × 100.

Ammonium Sulfate Precipitation of Cytosolic Factor. Aliquots of cytosol in 1.5-mL microfuge tubes were adjusted with saturated $(NH_4)_2SO_4$ (pH = 8.5 at 0 °C) to 40% saturation. After 1 h, the crude factor was pelleted (TOMY MTX-150 refrigerated microfuge, 12000*g*/10 min), resuspended in TAPS buffer to the original starting volume, and used immediately.

Heat Treatment of Factor Preparations. Factor preparations of ≤500 μL in 1.5-mL microfuge tubes were heated in a boiling H_2O bath for 10 min and then centrifuged at 12000*g*. The supernatant was removed and used immediately.

Fractionation on Centricon Microconcentrators. Just before use, each Centricon device was rinsed with 500 μL of TAPS buffer by centrifugation (2000*g*/30 min). For experiments employing C-10 units, 500 μL of sample was loaded, followed by centrifugation (2000*g*/30 min). Centricon C-3 devices required a 60-min spin at 5500*g*. After centrifugation, the sample in the top chamber was adjusted to the original volume with TAPS buffer. This sample and the filtrate were used immediately. The molecular weight cutoffs of the Centricon microconcentrators (10000 for C-10 and 3000 for C-3) were confirmed with the following standards; cytochrome *c* (MW = 12500), glucagon (MW = 3500), and vitamin B_{12} (MW = 1350).

Incubation of Nuclear Extract with Degradative Enzymes. Heat-treated nuclear extract (500 μL) was incubated with either RNase A, DNase I, or trypsin according to manufacturers' instructions for 1 h at 4 °C. Nuclear extract was also incubated with Pronase for 1 h at 50 °C. The enzymes were then removed by fractionation on a Centricon C-10 microconcentrator. Aliquots (100 μL) of the filtrate were desalted on the mini-Sephadex G-10 columns and assayed immediately for DNA/DEAE binding.

Results

Comparison of Activated Glucocorticoid Complex Binding to Nuclei and DNA before and after Sephadex G-25 Chromatography. Consistent with our earlier results (Simons & Miller, 1984), approximately half of the initial receptor–steroid complexes activated by heat and dilution are able to bind to either nuclei or DNA/DEAE minicolumns (Table IA). Little difference in binding was observed between crude nuclei and nuclei washed free of cytoplasmic contaminants.

Table II: Sephadex G-25 Chromatography of Unactivated and Activated Complexes[a]

sample	% complexes bound to DNA
unactivated cytosol	21
cytosol activated by heat and dilution	76
cytosol activated by Sephadex G-25 chromatography	47
cytosol activated by heat and dilution, then Sephadex G-25 chromatographed	45

[a] Activated cytosol was prepared as described under Experimental Procedures; unactivated cytosol was prepared in the same manner with the omission of the heat and dilution step. Fifty microliters of each sample before and after Sephadex G-25 chromatography was then assayed for DNA binding in the minicolumn assay. The percent of recovered complexes that bound to DNA–cellulose was determined as in Table IB.

Removal of free steroid from activated complexes by Sephadex G-25 chromatography also provided a direct quantitation of total complexes. Using this value, we found that a slightly higher percentage of added complexes bound to nuclei after Sephadex G-25 chromatography (Table IA). In contrast, the DNA binding of chromatographed complexes was significantly decreased. When the DNA binding is expressed as the percentage of the recovered complexes, i.e., those that bound to the DNA and the HAP (or DEAE) of the minicolumns, this decrease was even more dramatic (Table IB). All subsequent results are expressed in the manner of Table IB since it normalizes the data to the actual number of detected complexes.

Activated complexes that had been chromatographed on Sephadex G-10 bound to DNA almost as well as nonchromatographed complexes (data not shown). The use of resins able to include larger molecules than Sephadex G-25 (i.e., G-50), or even two sequential Sephadex G-50 gel filtration columns, did not further reduce the percentage of activated complexes that bound to DNA (data not shown). This suggests that a macromolecular cytosolic substance is required for maximal DNA binding.

Effect of Gel Filtration on Unactivated and Activated Complexes. Sephadex G-25 chromatography is known to cause activation of glucocorticoid complexes (Cake et al., 1976; Bailly et al., 1978). As shown in Table II, the percent of total complexes that bound to DNA after G-25 chromatography was the same whether activated or unactivated cytosol was used.

Large variations in activated complex binding were sometimes observed with different lots of HAP. To alleviate this problem, DEAE-cellulose was substituted for HAP in all subsequent minicolumn assays. DEAE-cellulose could be used instead of HAP since our receptor preparations do not contain meroreceptors (Reichman et al., 1984). Another source of variation was the Sephadex G-25, possibly due to different flow rates of commercially prepared columns. Since Sephadex G-50 gave much more reproducible results and removes a wider range of low molecular weight molecules, all future experiments were performed with this matrix.

Reconstitution of DNA Binding of Factor-Depleted Receptor–Steroid Complexes. If the DNA binding of activated complexes is factor-mediated, than the readdition of factor to factor-depleted complexes should restore full DNA-binding activity. Our source of factor was activated, competed cytosol which had been incubated with 2.5×10^{-5} M [^{1}H]Dex to

Table III: Reconstitution of DNA Binding of Activated Complexes after Gel Filtration Chromatography

sample	addition	% complexes bound to DNA expt 1	expt 2
activated cytosol	buffer	78	80
activated cytosol	competed cytosol	76	
activated cytosol through Sephadex G-50	buffer	46	38
activated cytosol through Sephadex G-50	competed cytosol	74	78
activated cytosol through Sephadex G-50	competed cytosol through Sephadex G-50	43	
activated cytosol through Sephadex G-50	10 mM DTT		46

[a] HTC cytosolic receptors labeled with [^{3}H]Dex, or [^{1}H]Dex for competed cytosol, were activated as described under Experimental Procedures and chromatographed on Sephadex G-50 columns. Equal volumes of the two cytosols (or buffer ± DTT) were mixed together and assayed on DNA/DEAE minicolumns. The amount of binding to DNA was determined as in Table IB.

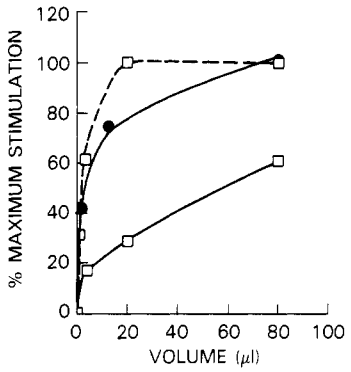

FIGURE 1: Comparison of factor concentration in nuclear extract and competed cytosol. Indicated volumes of nuclear extract and competed cytosol were added to 40 μL of factor-depleted cytosol and assayed on DNA/DEAE minicolumns as described under Experimental Procedures. (—●—) Factor from nuclear extract; (—□—) factor from cytosol; (---□---) cytosolic factor concentrated 10-fold by $(NH_4)_2SO_4$ precipitation.

occupy all of the receptors and to block exchange binding with [^{3}H]Dex. When competed cytosol was added to factor-depleted cytosol, full DNA-binding ability was restored (Table III). As expected, when the factor source (competed cytosol) was chromatographed on Sephadex G-50, it lost the ability to reconstitute DNA binding. While free -SH groups have been found to be important for DNA binding of receptor–steroid complexes (Bodwell et al., 1984; Tienrungroj et al., 1987a; Miller & Simons, 1988), the cytosolic factor is not a small, nonspecific reducing agent since DTT did not cause any significant increase in the DNA binding of chromatographed complexes (Table III). The addition of more factor in competed cytosol did not increase the DNA-binding ability of nonchromatographed activated cytosol (Table III).

A Nuclear Pellet Extract Can Reconstitute DNA Binding. Since the above cytosolic factor increased the DNA binding of Sephadex G-50 chromatographed, activated complexes (Table III) but was not required for nuclear binding (Table I), it seemed reasonable that nuclei would also contain this factor. As shown in Figure 1, both competed cytosol and a 0.5 M NaCl extract of crude nuclei reconstituted DNA binding in a dose-dependent manner. Since the nuclear pellet extract was prepared so that the volume of extract was essentially equal to the volume of cytosol obtained from the same number of cells, it can be calculated that the nuclear extract contains 5–10 times more factor activity than does the cytosol.

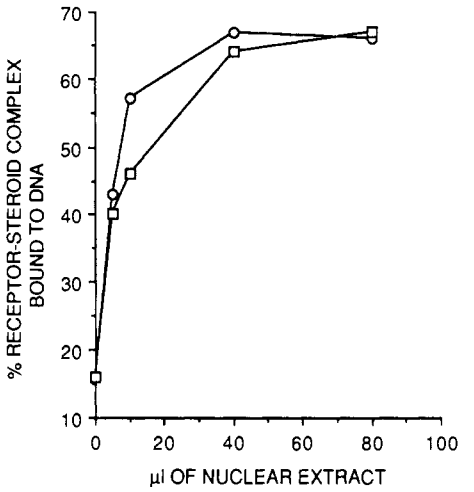

FIGURE 2: Reconstitution of the DNA-binding activity of receptor–steroid complexes in DNA–cellulose column flow-through with nuclear extract factor. Activated G-50-chromatographed [^{3}H]Dex-bound receptors (500 μL) were loaded onto a 2.5-mL DNA–cellulose column and washed with TAPS buffer. Aliquots (100 μL) of the flow-through were mixed with nuclear extract and assayed on minicolumns for DNA binding. Nonspecific binding to cellulose was determined from the binding to cellulose/cellulose and to DNA–cellulose/cellulose minicolumns and was subtracted from the binding to DNA/DEAE minicolumns before calculating the percent of recovered complexes that bound to DNA as described under Experimental Procedures. (○) Nuclear extract desalted by chromatography on Sephadex G-10; (□) nuclear extract desalted on Sephadex G-10, followed by heating.

However, neither preparation caused all of the complexes in activated cytosols to bind to DNA either before or after Sephadex chromatography (data not shown).

Reconstitution of DNA-Binding Activity for Complexes in the Flow-Through of DNA–Cellulose Columns. Activated, Sephadex G-50 chromatographed complexes were then passed over a DNA–cellulose column to remove those complexes that still bound DNA. The complexes that did not bind DNA were collected in the flow-through. The addition of nuclear extract factor to these complexes greatly stimulated their ability to bind to DNA (Figure 2). As with unfractionated activated cytosols, all of the complexes could not be stimulated to bind to DNA.

Properties of the DNA-Binding Activity Factor in Cytosol and Nuclear Pellet Extracts. Factor in both cytosol and extracts of crude nuclei could be pelleted by the addition of $(NH_4)_2SO_4$ to 40% saturation (Table IV). Higher concentrations of salt did not increase the yield of factor, and factor partially purified by heat precipitation is no longer $(NH_4)_2SO_4$ precipitable. Factor could not be precipitated by 10% trichloroacetic acid and/or be recovered from the TCA pellets (data not shown). While the salt-precipitated factor was stable to repeated freezing and thawing, storage of the $(NH_4)_2SO_4$-precipitated nuclear extract in buffer solution at 4 °C for 16 h did result in some loss of factor activity (data not shown). The factor was extremely stable to heat in the crude extracts (Figure 2 and Table IV).

The factor in heated cytosols and nuclear extracts was still found in the excluded volume of the Sephadex G-10 columns (Table IV). This indicates that the factor is not a very small molecule that is normally associated with other larger macromolecules. Thus the minimum molecular weight of the cytosol and nuclear factor is ~700. The maximum molecular weight was determined by centrifuging factor preparations of heated nuclear extract and cytosol through filtration devices with known molecular weight cutoffs. The factor from both sources passed through a Centricon C-10 membrane (MW

Table IV: Summary of Characteristics of DNA-Binding Activity Factors

factor treatment	% of DNA-binding activity seen after treatment of factor in nuclear extract	cytosol
$(NH_4)_2SO_4$ ppt in crude preparations	103	100
$(NH_4)_2SO_4$ ppt in partially purified preparations	46	ND
heat stable (100 °C/10 min)	100	104
stable to freeze–thaw	100	100
RNase (500 μg/mL)	97	ND
DNase (500 μg/mL)	84	ND
Pronase (200 μg/mL)	77	ND
trypsin (100 μg/mL)	68	ND
binds to DNA	no	no (yes)
passes through Centricon C-3	90	71
excluded by		
Sephadex G-10	120	85 (yes)
Sephadex G-25	4	18 (ND)
Sephadex G-50	0	0 (no)
Sephadex G-10 after 100 °C/10 min	77	76 (ND)

[a] Comparison of factor from competed cytosol and nuclear extract. To determine the percent of DNA-binding activity, the increase in DNA binding caused by the addition of factor was divided by the difference in DNA binding between activated complexes and factor-depleted complexes. The items in parentheses apply to the second, less abundant, larger molecular weight factor found in crude cytosol. ND = not determined.

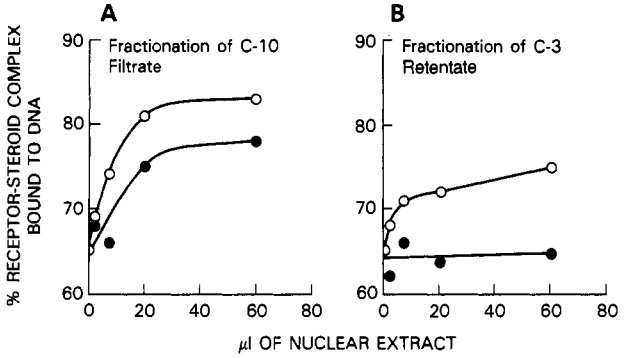

FIGURE 3: Fractionation of nuclear extract factor on Centricon C-3 microconcentrators. (A) Desalted nuclear extract was heated at 100 °C for 10 min. The mixture was centrifuged to remove precipitated protein and the supernatant fractionated on a Centricon C-10 microconcentrator. Eight hundred microliters of this filtrate was then fractionated on a Centricon C-3 microconcentrator. The material that was retained in the top chamber of the microconcentrator was diluted up to the original volume with TAPS buffer. Aliquots of both the C-3 filtrate (○) and diluted retentate (●) were assayed for factor activity as described under Experimental Procedures. (B) The reconstituted retentate of panel A was loaded onto a second C-3 microconcentrator and refractionated. The retentate was again diluted up to original volume with TAPS buffer. Aliquots of filtrate (○) and diluted retentate (●) were assayed for factor activity as in panel A.

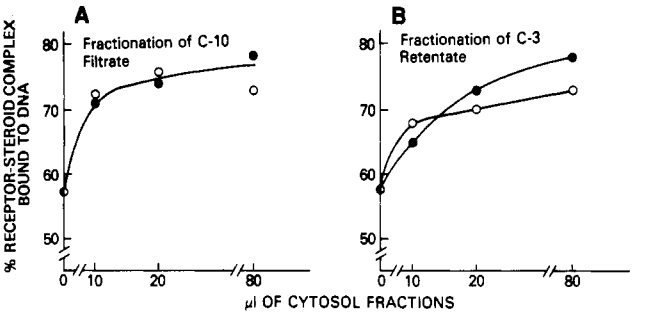

FIGURE 4: Fractionation of cytosolic factor on Centricon C-3 microconcentrators. (A) Cytosol (diluted 1:2 with TAPS buffer) was heated at 100 °C for 10 min, and the precipitated protein was removed by centrifugation. One milliliter of supernatant was then fractionated on a Centricon C-10. Five hundred microliters of filtrate was then fractionated on a Centricon C-3 microconcentrator, and aliquots of filtrate (○) and diluted retentate (●) were assayed for factor activity as described in Figure 3A. (B) Reconstituted retentate of panel A was refractionated on a second C-3 microconcentrator and aliquots of filtrate (○) and diluted retentate (●) were assayed for factor activity as in panel A.

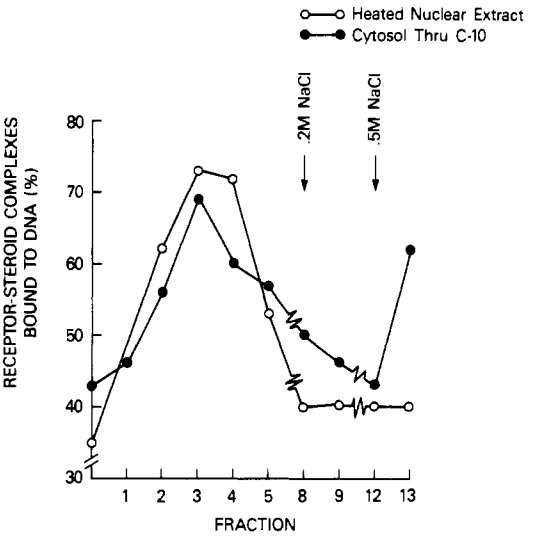

FIGURE 5: Chromatography of factor on DNA–cellulose. A 350-μL aliquot of factor preparation was loaded onto a 1-mL DNA–cellulose column in a 3-mL syringe. The column was washed successively with 3 mL of TAPS buffer, 3 mL of 0.2 M NaCl in TAPS buffer, and 2 mL of 0.5 M NaCl in TAPS buffer. Fractions (350 μL) were collected, and the conductivity was determined. The first two fractions of each step elution which showed conductivity measurements equal to the added salt were assayed for factor activity (after desalting on Sephadex G-10 columns). Arrows indicate changes in conductivity due to the higher salt elutions; other fractions were collected at each salt concentration but not assayed. (○) Factor from heat-treated nuclear extract which had been desalted on Sephadex G-10; (●) factor from competed cytosol that was diluted 1:2 with TAPS buffer and filtered through a Centricon C-10 microconcentrator.

cutoff 10000) and was recovered in the filtrate (data not shown). The factor in each C-10 filtrate also passed through a Centricon C-3 membrane (MW cutoff 3000, Figures 3A and 4A). The material remaining in the top of each Centricon C-3 (designated the retentate, ~20% of the original sample volume) was reconstituted with buffer to the initial sample volume and then refractionated on a second Centricon C-3 in order to achieve a more complete separation of small molecules. With the nuclear extract, almost all of the factor was found in the filtrate of the second C-3 microconcentrator (Figure 3B). This strongly argues that the DNA-binding factor in the nuclear extract has a maximum molecular weight of 3000. With cytosol, about equal amounts of factor were found in both the filtrate and retentate of the second C-3 microconcentrator (Figure 4B). This indicates that cytosol contains at least two factors. The smaller sized factor appears to have the same molecular weight (≤3000) as the nuclear factor. From an analysis of Figure 4, it can be calculated that about 70% of the DNA-binding activity in cytosol is due to this small factor. The second cytosolic factor has an apparent molecular weight of 3000–10000.

DNA–Cellulose Chromatography of Factor Preparations. Since nuclear extracts contain high concentrations of the DNA-binding activity factor, we used DNA–cellulose chromatography to determine whether either the nuclear or cytosolic factor binds to DNA. No factor activity in nuclear extracts was retained on DNA–cellulose columns (see Figure 5). Similarly, the majority of DNA-binding activity of the

Centricon C-10 filtrate of cytosol did not bind to the DNA–cellulose (Figure 5). The amount of factor activity that eluted with 0.5 M NaCl was small since, in other experiments, the activity in the subsequent fractions quickly dropped back to base-line values (data not shown). This high-salt peak of activity was not seen with the nuclear extracts.

Incubation of Nuclear Extract with Degradative Enzymes. The activity of nuclear factor was not eliminated by digestion with any of the enzymes examined (Table IV). Analysis of the RNase and DNase digestions on agarose gels, followed by staining with ethidium bromide, indicated that RNA and DNA were completely digested. When the Pronase digestion products were visualized on a 15% denaturing SDS–polyacrylamide gel with Coomassie blue (Simons, 1987), a lightly staining broad band of protein with a maximum molecular weight of 6000 was visible. Hence, while the vast majority of protein was digested, Pronase was not capable of digesting the factor preparations to single amino acids under our incubation conditions. Similarly, the inability of Pronase or trypsin to cause more than a slight reduction in factor activity (Table IV) could be due to a resistance of the factor to these proteases. These data also show that the factor is not just a nonspecific, low molecular weight degradation product of nuclear proteins. Thus the factor(s) may be a peptide, but it does not appear to be DNA or RNA.

Discussion

Comparison of the nuclear and DNA binding of Sephadex-chromatographed, activated receptor–steroid complexes has revealed the existence of a new factor which is involved in the expression of glucocorticoid receptor activity. This new factor is intracellular, is required for the maximal binding of complexes of DNA, and can be removed from solutions of activated complexes by Sephadex G-25 or G-50 chromatography (Tables I–III). The presence of this factor is supported by the fact that cytosol, in which all of the receptors were either blocked by [^{1}H]Dex (Table III) or inactivated by heating (Table IV), will restore the original DNA-binding activity to activated complexes depleted of this factor. However, no reconstitution was seen with cytosol that had been chromatographed on Sephadex G-50 (Table III). High-salt extracts of crude nuclear pellets were a 5–10 times more abundant source of the same activity (Figure 1). Fractionation of the cytosolic and nuclear factors on Centricon C-3 microconcentrators (Figures 3 and 4) and DNA–cellulose columns (Figure 5) indicated that there are two components in the cytosol but just one component in the nuclear extract. We can see no difference between the factor in nuclear extracts and the predominate factor in cytosol (Table IV). We therefore conclude that the nuclear and the major cytosolic factor are the same species. We have not yet purified the nuclear factor, but we believe that all of the activity is due to just one molecular species. It appears highly unlikely that two or more molecules with the above ability to augment the DNA binding of activated complexes could have all of the properties listed in Table IV. The data also suggest that the factor is not DNA or RNA and may not be proteinaceous. However, the possibility that the factor is one of these species but is resistant to enzymic degradation cannot be excluded.

Identification of factor activities is fraught with artifacts. Some salts [i.e., ≥12 mM NaCl or ≥5 mM $(NH_4)_2SO_4$] and proteins (i.e., aprotinin) mimicked the effects of factor and caused increased DNA binding (data not shown). Thus all salts were first removed by Sephadex G-10 columns when factor from nuclear extracts or fractions from DNA–cellulose columns were assayed. Likewise, the use of protease inhibitors to study DNA binding of receptor fragments (Reichman et al., 1984; Rehmus et al., 1987) should be conducted with care. The ability of exogenous substances to mimic the effects of our factor does complicate our study but in no way diminishes the importance and ability of an endogenous substance in HTC cells to modulate the DNA-binding capacity of activated complexes.

That Sephadex G-25 chromatography causes a decrease in the DNA binding of activated complexes is of major importance because (1) such procedures have been used for many years to remove small molecules from preparations of activated glucocorticoid complexes [e.g., Higgins et al. (1973)] and (2) Sephadex G-25 chromatography has been used to *activate* glucocorticoid complexes [e.g., Ali and Vedeckis (1987) and Bodine and Litwack (1988b)]. The fact that the same amount of complexes will bind to DNA whether or not the complexes are activated before chromatography indicates that Sephadex G-25 chromatography can simultaneously cause activation of the complexes and depletion of the factor (Table II and following paper in this issue).

Several other factors have been described that increase the DNA binding of activated glucocorticoid complexes. All of these factors are larger than our factor [i.e., ~72K (Payvar & Wrange, 1987), 70K (Tymoczko et al., 1988), 10–12K (Grippo et al., 1985; Tienrungroj et al., 1987b), and >5K (Schmidt et al., 1985; Harmon et al., 1988)], and none were isolated from crude nuclei. Small molecular weight substances, like β-mercaptoethanol and Zn^{2+} (Sabbah et al., 1987), that increase the DNA binding of activated complexes cannot be our factor since these compounds would be included in Sephadex G-10 columns and since added thiols did not cause increased DNA binding (Table III). Our factor may, however, be the same as that implicated by Peleg et al. (1988) to explain why some preparations of the progesterone B receptor display poor DNA binding but much more nuclear binding. In addition, Edwards et al. (1989) have observed a nuclear activity that increases the DNA binding of progesterone receptors. Thus, multiple DNA-binding activity factors may exist within the same system. In fact, HTC cytosol does contain a second, minor DNA-binding activity factor which binds to DNA–cellulose (Figure 5) and has a molecular weight of 3–10K (Figure 4). The physical properties of this minor cytosolic factor are suspiciously similar to those factors described by three other laboratories (Grippo et al., 1985; Schmidt et al., 1985; Harmon et al., 1988).

The present results clearly demonstrate that DNA–cellulose and nuclei do not always detect the same population of receptor–steroid complexes in activated cytosols (see Table I). The abundance of our factor in nuclei nicely explains why Sephadex chromatography had previously been found to decrease the DNA, but not nuclear, binding of activated complexes (Simons, 1977; Tienrungroj et al., 1987b). Thus the involvement of binding factors can be easily overlooked if only the binding to DNA is examined. Both the predominant nuclear localization of the factor and its ability to increase the DNA binding make this factor a biologically relevant component in the nuclear action of glucocorticoid receptors.

The fact that a major portion of activated complexes bind to DNA after Sephadex G-50 chromatography without added factor argues that activated complexes are not homogeneous but consist of at least two populations, one of which requires factor for DNA binding to occur (see Figure 2). Support for this conclusion comes from the observations that most preparations of purified, unactivated receptor–glucocorticoid complexes cannot be activated to the same extent as crude

complexes (Idziorek et al., 1985; Hapgood & von Holt, 1987; Denis & Gustafsson, 1989) unless a cytosolic preparation is also added (Grandics et al., 1984). Similarly, activation of crude glucocorticoid complexes by dialysis was found to be less effective than by heating (Sato et al., 1980), and Bio-Gel chromatographed, unactivated complexes could not be further activated by heat treatment (Grippo et al., 1986). Both of these latter results would be predicted since dialysis (and Bio-Gel chromatography) would also remove our low molecular weight cytosolic factor that is required for maximal DNA binding. Further evidence that activated complexes are heterogeneous and that only a subpopulation respond to our factor is presented in the following paper in this issue (Cavanaugh & Simons, 1990).

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Glucocorticoid Receptor Binding to Calf Thymus DNA. 2. Role of a DNA-Binding Activity Factor in Receptor Heterogeneity and a Multistep Mechanism of Receptor Activation

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ABSTRACT: In the preceding paper [Cavanaugh, A. H., & Simons, S. S., Jr. (1990) *Biochemistry* (preceding paper in this issue)], we characterized an apparently identical factor in the cytosol and the nuclear extract of HTC cells that is required for the DNA binding of approximately 43% of the activated receptor–glucocorticoid complexes. In the present study, both those activated complexes that are influenced by this factor and the role of this factor in the process of activation are examined. We find that sodium arsenite inhibits only the DNA binding of those complexes that require factor. Conversely, methyl methanethiolsulfonate inhibits the DNA binding of only those complexes that are independent of factor. These results provide direct chemical evidence for two populations of activated complexes. Double-reciprocal plots revealed that the increase in DNA binding with endogenous factor occurred by recruiting new complexes for DNA binding as opposed to increasing the binding affinity of existing complexes. These results further suggest that factor associates only with the receptor–steroid complex and does not additionally interact with DNA. A saturable association of factor with complexes was indicated since the amount of available factor in cytosolic solutions decreased after activation of the complexes. Sodium molybdate is known to inhibit the activation of HTC cell receptor–steroid complexes. When factor was added to complexes that had been subjected to activating conditions in the presence of the inhibitor sodium molybdate, no increased DNA binding was observed. In contrast, the addition of factor to Sephadex G-50 chromatographed, activated complexes did stimulate DNA binding, even in the presence of molybdate. Collectively, these data establish the presence of two populations of activated receptor–steroid complexes. Activation of one population of complexes was shown to involve an additional step that is not seen for the other population. This additional step is characterized by an insensitivity to molybdate and a requirement for our recently characterized DNA-binding activity factor.

Activation of receptor–steroid complexes, whereby the initially formed complexes attain a high affinity for DNA, is still a poorly understood process (Baxter et al., 1972; Kalimi et al., 1975; Milgrom, 1981). Progress has been impeded both by the lack of a rigorous definition of activation and by the apparent involvement of several nonreceptor components, only some of which have been studied in any detail (Schmidt & Litwack, 1982; Harmon et al., 1984, 1988; Schmidt et al., 1985; Tai et al., 1986; Webb et al., 1986; Pratt, 1987; Bodine & Litwack, 1988a,b; Mechinchi et al., 1988). One major advance has been the discovery that sodium molybdate can block activation [Leach et al., 1979; for review see Dahmer et al. (1984)]. In the preceding paper in this issue (Cavanaugh & Simons, 1990), we described and characterized a macromolecular factor that is required for the maximal binding of glucocorticoid receptors to DNA. This factor was first identified in the cytosol of HTC cells by the observation that Sephadex G-25 or G-50 gel filtration chromatography decreased the ability of activated receptor–[^{3}H]dexamethasone (Dex)[1] complexes to bind to calf thymus DNA–cellulose. This decrease could be reversed by the addition of cytosolic components. This decreased binding was not observed when the same chromatographed complexes were tested for their ability to bind to isolated nuclei. We subsequently found that salt extracts of crude nuclei contained 5–10 times more of the apparently identical DNA-binding activity factor. Hence the removal of cytosolic factor during the chromatography of activated complexes caused a decrease in the binding to DNA but not to nuclei since the nuclear binding assay supplied an

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[1] Abbreviations: Dex or dexamethasone, 9α-fluoro-16α-methyl-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; TAPS, 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonic acid; MMTS, methyl methanethiolsulfonate.