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Factor-assisted DNA Binding as a Possible General Mechanism for Steroid Receptors. Functional Heterogeneity Among Activated Receptor–Steroid Complexes

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We previously reported that activated glucocorticoid receptor–steroid complexes from rat HTC cell cytosol exist as at least two sub-populations, one of which requires a low molecular weight (700–3000 Da) factor(s) for binding to DNA. This factor is removed by Sephadex G-50 chromatography and is found predominantly in extracts of crude HTC cell nuclei. We have now determined that factor is not limited to HTC cells since an apparently identical factor(s) was found in nuclear extracts of rat kidney and liver as well as human HeLa and MCF-7 cells. Furthermore, the DNA binding of a sub-population of human glucocorticoid receptors depends on factor. While these results were obtained with agonist (dexamethasone) bound receptors, a sub-population of HTC cell receptors covalently labeled by the antiglucocorticoid dexamethasone 21-mesylate also displayed factor-dependent DNA binding. This receptor heterogeneity was not an artifact of cell-free activation since the cell-free nuclear binding of dexamethasone mesylate labeled complexes was, as in intact cells, less than that for dexamethasone bound complexes. Earlier results suggested that the increased DNA binding with factor involved a direct interaction of receptor with factor(s). We now find that the factor-induced DNA binding is retained by amino terminal truncated (42 kDa) glucocorticoid receptors from HTC cells. Thus the ability of receptor to interact with factor(s) is encoded by the DNA and/or steroid binding domains. Two dimensional gel electrophoresis analysis of dexamethasone-mesylate labeled 98 kDa receptors revealed multiple charged isoforms for both sub-populations but no differences in the amount of the various isoforms in each sub-population. Finally, activated progesterone and estrogen receptor complexes were also found to be heterogeneous, with a similar, if not identical, small molecular weight factor(s) being required for the DNA binding of one sub-population. The observations that functional heterogeneity of receptors is not unique to glucocorticoid receptors, whether bound by an agonist or antagonist, and that the factor(s) is neither species nor tissue specific suggests that factor-assisted DNA binding may be a general mechanism for all steroid receptors.

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INTRODUCTION

The DNA binding of receptor–steroid complexes is dramatically increased by a process called activation [1–3] or transformation [4]. Despite many years of investigation, the exact mechanism of activation is unknown. While originally postulated to be one step [5], activation of receptor–glucocorticoid complexes has subsequently been proposed to be a multi-step process [6–11]. Activation *in vitro* can be achieved by a number of methods including dialysis [12], dilution, heating, altering the pH, salt precipitation, RNase, and ATP [3]. Numerous laboratories have reported the existence of endogenous regulators believed to either inhibit or stimulate this process (for review see Ref. [13]). The most well characterized inhibitor of activation is a low molecular weight molecule (< 1500 Da) termed modulator, first described by Cake *et al.* [14], and later purified and characterized by Bodine and Litwack as a novel phosphoglyceride [10, 15, 16]. A similar factor has been described by Meshinchi *et al.*

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[17], which is stable to heating at 220°C and copurifies with endogenous molybdenum [18]. Both factors mimic molybdate in their effects on receptor function but their physicochemical properties are quite different. At least two laboratories have described macromolecular stimulators of the activation process for glucocorticoid receptors. Schmidt *et al.* [19] reported a protein whose activity can be mimicked by RNase A or the RNase A S peptide while Tymoczko *et al.* [20] described a larger protein (M_w 70 kDa) which stimulated receptor–steroid complex activation.

Factors which influence the binding of previously activated glucocorticoid complexes to either DNA or nuclei have also been reported. Cake *et al.* [21] showed that pyridoxal phosphate directly inhibits DNA binding. Payvar and Wrange [22] found a 72 kDa protein associated with purified receptor–steroid complexes which may be involved with the recognition of specific sequences of DNA. Several laboratories have described species $\geqslant$5 kDa which increase DNA binding [6, 7, 23].

Many members of the superfamily of hormone-regulated DNA-binding proteins are thought to require auxiliary factors for maximal DNA binding to their response elements [24]. These include the thyroid receptors, for which binding to thyroid response elements was significantly enhanced by the addition of a 65 kDa protein [25]. Similarly, the interaction between the vitamin D receptor and its response element was markedly enhanced by an ~55 kDa nuclear factor [26]. Recently, a family of proteins has been described which enhance cellular responsiveness to retinoic acid and its metabolites [27]. These nuclear proteins, termed retinoid X receptors (RXR), heterodimerize with retinoic acid, thyroid and vitamin D receptors to enhance their DNA binding but do not interact with estrogen or glucocorticoid receptors [28]. However, the DNA binding of progesterone receptors has been found to require a >30 kDa nuclear protein(s) [29, 30] (D. Edwards, personal communication) and estrogen receptor complex binding to DNA can be increased by an ~45 kDa nuclear factor [31].

None of the above studies of regulators and factors have addressed the question of whether all activated complexes, or only a sub-population of activated complexes, are affected. Recently, we have identified and partially characterized a low molecular weight factor from rat hepatoma tissue culture (HTC) cells that is required for approx. 40% of the activated glucocorticoid receptor–steroid complexes to bind to calf thymus DNA [11, 32]. This factor has an apparent M_w of 700–3000, is removed by Sephadex G-50 chromatography, and is heat stable, salt precipitable, and resistant to digestion by RNase A and DNase I. The factor itself does not bind to DNA or change the affinity of receptor–steroid complexes for DNA. Instead the data suggests that the factor associates with some receptors only after their activation to permit DNA binding. Further support for the action of this factor on one sub-population of activated complexes came from studies with various sulfhydryl reagents. The DNA binding of those complexes dependent on factor was selectively inhibited by sodium arsenite, while the DNA binding of factor-independent complexes was specifically blocked by methyl methanethiolsulfonate (MMTS) [11].

The purpose of the present study was to determine whether the phenomenon of factor-assisted DNA binding by a sub-population of receptor–steroid complexes was unique to the agonist-bound complexes of HTC cells or could be seen with an antiglucocorticoid, in other cell types, and with other receptors. We now report that an apparently identical DNA binding activity factor(s) is found in nuclear extracts from rat kidney and liver cells as well as from human HeLa and MCF-7 cells. In addition, glucocorticoid receptors from HeLa cells exist as a heterogeneous population that is indistinguishable from those of HTC cells. Furthermore, the activated complexes of agonist and antagonist steroids were equally affected. Finally, activated estrogen and progesterone receptor–steroid complexes were also shown to be heterogeneous in that maximal DNA binding required the presence of a small molecular weight factor with the same characteristics as the HTC cell nuclear factor.

EXPERIMENTAL

Materials

[1,2,4,-^{3}H]Dexamethasone (Dex) (36.5 Ci/mmol) was purchased from Amersham. [6,7-^{3}H]Dexamethasone 21-mesylate (Dex-Mes) (44.7 Ci/mmol), [2,4,6,7-^{3}H]estradiol (108.8 Ci/mmol), and [17α-methyl-^{3}H]promegestone, (R5020) (86 Ci/mmol) were obtained from NEN Dupont. HTC cells were grown in Swim's S-77 media supplemented with 5% newborn calf serum, 5% fetal bovine serum and 0.03% glutamine as described previously [33]. HeLa cells (supplied by Norman Cooper, NIAID/NIH) were grown in Eagle's spinner media supplemented with 5% horse serum and 0.03% glutamine. MCF-7 cells were grown in Richter's improved minimal essential medium (zinc option with folic acid), supplemented with 10% fetal bovine serum and 0.03% glutamine. Kidneys and livers were obtained from male Sprague–Dawley rats 3 days after adrenalectomy. Calf uterine tissue from a 2-month-old calf was supplied by the NIH Animal Center (Poolesville, MD). TAPS 8.8 buffer consisted of 25 mM TAPS, 1 mM EDTA, and 10% glycerol (pH 8.8 at 4°C). Hepes buffer contained 25 mM Hepes, 1 mM EDTA and 10% glycerol (pH 7.5 at 25°C). Tris buffer consisted of 25 mM Tris, (pH 7.5 at 25°C) 12 mM monothioglycerol (Sigma), 10% glycerol. DNA–cellulose and Sephadex G-10 and G-50 were obtained from Pharmacia. Sodium arsenite was from Baker and methyl methanethiolsulfonate from Aldrich. Centricon C-3 ultrafiltration devices were supplied by Amicon Corp.

Isolation of steroid receptors

The preparation of cytosolic glucocorticoid receptors from freeze–thaw lysed HeLa and HTC cells was as described previously [34]. Progesterone receptors were isolated from MCF-7 cells which had been washed three times with cold phosphate buffered saline and frozen in liquid nitrogen. An equal volume of Hepes buffer was added and the pellet thawed slowly on ice, followed by vigorous vortexing and centrifugation in a Sorvall RC-3B centrifuge at 3000 *g* for 10 min at 0°C. The nuclear extract was prepared from the pellet resulting from this spin (see below). The supernatant was termed cytosol and stored in liquid nitrogen. Supernatants from higher speed centrifugations displayed identical properties (data not shown) but were not used due to the lower yields.

Estrogen receptors were isolated from calf uterine tissue which was frozen in liquid nitrogen immediately after surgery. Frozen tissue was pulverized in a Fisher tissue pulverizer, suspended in 2 vol of ice cold Tris buffer, and homogenized at 4°C in a Brinkman polytron at a setting of 4 for three, 15 s bursts, with 30 s of cooling between bursts. The homogenate was centrifuged in a Sorval RC-5B centrifuge at 32,000 *g* for 15 min at 0°. Nuclear extracts were made from the pellet resulting from this spin (see below). The supernatant was spun at 100,000 *g* for 1 h at 4°C in a Beckman L8-70 ultracentrifuge. The supernatant from this spin was termed cytosol and was stored in liquid nitrogen.

Preparation of activated receptor–steroid complexes

Glucocorticoid receptors were bound with 5×10^{-8} M [^{3}H]Dex ± a 500-fold excess of non-radioactive Dex for 2.5 h and the total receptor–steroid complexes determined after the addition of dextran coated charcoal (DCC) as described [35]. Receptor–steroid complexes were activated by adding an equal volume of TAPS 8.8 buffer and incubating at 20°C for 30 min. Covalently labeled complexes were prepared by incubation with 1.5×10^{-7} M [^{3}H]Dex-Mes ± a 100-fold excess of non-radioactive Dex and quantitated by the slice-and-counting of complexes separated on SDS-polyacrylamide gels [34].

Progesterone receptors were incubated with 8.8×10^{-9} M [^{3}H]R5020 + 1.7×10^{-6} M of non-radioactive Dex ± 1000 × non-radioactive R5020 in Hepes buffer for 2.5 h at 4°C. Activation was achieved by heating the receptor–steroid complexes at 20°C for 30 min without dilution.

Estrogen receptors were incubated with 1.5×10^{-8} M [^{3}H]estradiol ± 1000 × non-radioactive estradiol in Tris buffer. Activation was achieved by heating at 20°C for 45 min without dilution.

Preparation and enrichment of nuclear factor(s)

The preparation of nuclear extract factor from cell pellets has been described previously [32]. Briefly, crude nuclei were extracted with 0.5 M NaCl and the extract concentrated by precipitation with 40% $(NH_4)_2SO_4$, followed by heating at 100°C and filtration through a Centricon C-3 ultrafiltration device. In some cases the supernatant from the $(NH_4)_2SO_4$ precipitation was assayed for factor activity. All factor preparations were desalted on Sephadex G-10 before use.

Activated complex binding to DNA/DEAE

The percentage of each receptor–steroid complex that could bind to DNA was quantitated by a modification of the DNA/DEAE mini-column procedure of Holbrook *et al.* [36] as described elsewhere [32] under conditions where DNA was in large excess. Single samples were loaded onto mini-columns and washed with TAPS 8.8 for glucocorticoid receptors, Hepes buffer for progesterone receptors, and Tris buffer for estrogen receptors. Cellulose blanks were included in most assays for HeLa cell glucocorticoid receptors and for progesterone receptors. These values (background) were subtracted from each DNA or DEAE value before the percentage of bound complexes was determined.

Chromatography of Dex-Mes labeled receptor–steroid complexes on DNA–cellulose

Receptor–steroid complexes were loaded onto columns (1 × 2.5 cm) of DNA–cellulose (bed volume = 3 ml) that had been equilibrated in TAPS 8.8 buffer. The column was then washed with 20 ml of TAPS 8.8 and the complexes were eluted with 10 ml of 0.5 M NaCl in TAPS 8.8.

Electrophoresis

Two dimensional electrophoresis consisted of non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-polyacrylamide gel electrophoresis on a 7% gel in the second dimension [37]. The gels were impregnated with Enhance (NEN, Dupont), soaked in 10% polyethyleneglycol, dried and placed against Kodak XAR-5 film at −80°C for approx. 1 month.

Unless otherwise noted, all results described in this paper have been repeated at least 2 times.

RESULTS

DNA-binding activity factor(s) in rat tissues

The DNA binding of a sub-population of activated HTC cell glucocorticoid receptor–steroid complexes requires a small molecular weight factor(s) present in cytosol but much more abundant in nuclear extracts [11, 32]. The existence of a factor-dependent subpopulation of activated complexes is routinely established by the reduction in DNA binding following chromatography of activated complexes through Sephadex G-50, which removes the initially present factor(s) [32]. When HTC cell nuclear factor(s) is added to these chromatographed complexes, the DNA binding capacity of the unchromatographed complexes is regenerated. In order to determine whether the originally identified factor(s) is limited to HTC cells,

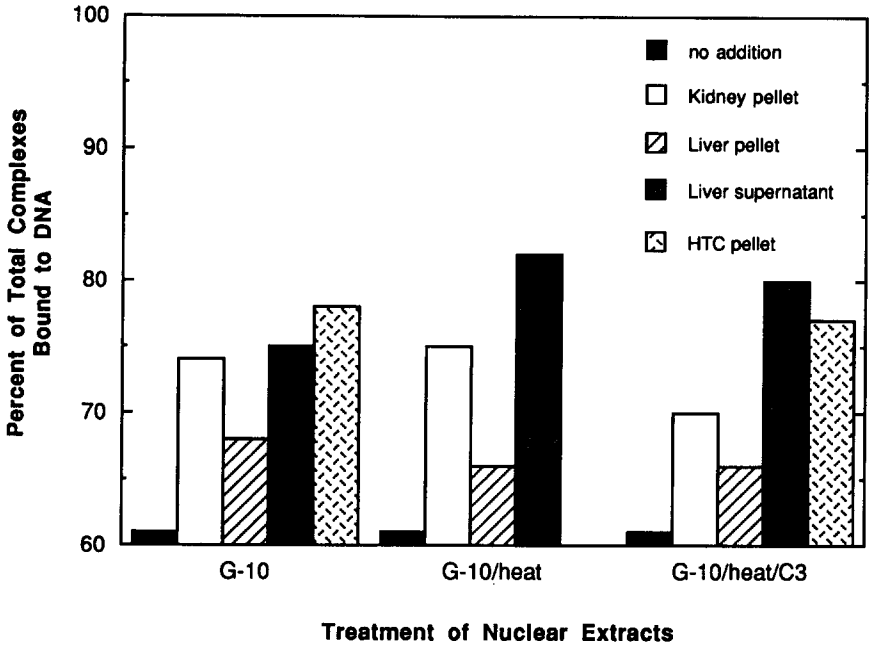

Fig. 1. Ability of various rat tissue nuclear extracts to increase the DNA binding of factor-depleted, HTC cell glucocorticoid receptor–steroid complexes. Crude nuclei were isolated from rat liver and kidney tissue and a nuclear extract prepared as described in Experimental. Aliquots (80 μl) of both the resuspended pellet and supernatant fractions of the $(NH_4)_2SO_4$ precipitation step in the enrichment of nuclear factor(s) were incubated with 40 μl of factor-depleted HTC activated cytosol and the extent of DNA binding assayed on DNA/DEAE mini-columns as described in Experimental. The percentage of complexes which bound to DNA was determined as follows: % total complexes bound to DNA = [dpm bound DNA/(dpm bound to DNA + DEAE)] × 100. 82% of the activated complexes initially bound to DNA. The activity of the $(NH_4)_2SO_4$ pellet for liver nuclei was determined once.

we asked whether a similar factor occurred in extracts of rat kidney and liver nuclei. Each extract was fractionated by the same procedure used to purify the DNA-binding activity factor from HTC cell nuclei. These steps included (1) precipitating with 40% $(NH_4)_2SO_4$, (2) desalting the resuspended pellet on a Sephadex G-10 column, followed by (3) heating the desalted preparation at 100°C for 10 min, after which the preparation was centrifuged to remove precipitated protein, and then (4) filtering the supernatant through a Centricon C-3 ultrafiltration device. Some factor activity was found in each source examined (Fig. 1). The small resuspended pellet resulting from the $(NH_4)_2SO_4$ precipitation of liver nuclear extracts had very low amounts of factor activity, possibly due to an incomplete precipitation of protein by the $(NH_4)_2SO_4$. For comparison, the liver supernatant had as much activity as factor from HTC nuclear pellet. The kidney nuclear pellet had measurable amounts of factor, but not as much as in the supernatant of liver extracts. With all tissues, factor activity survived boiling and filtration on the Centricon C-3 device, suggesting that the factor(s) is a heat stable species of <3 kDa. These properties are identical to those of the HTC cell nuclear factor(s) [32].

Heterogeneity in the DNA binding of glucocorticoid complexes from HeLa cells

Human glucocorticoid receptor–steroid complexes from HeLa cells, that were either unactivated, activated, or depleted of factor by chromatography on Sephadex G-50 after activation, were tested for their ability to bind DNA in the presence or absence of enriched nuclear factor(s) from either HeLa or HTC cells (Fig. 2). As expected, [11] only a small percentage of the unactivated complexes bound to DNA whether or not nuclear factor was added. When complexes were activated by heating at 20°C, $>80\%$ of the complexes bound to DNA, a percentage consistent with values seen with glucocorticoid receptors from HTC cells. The ability of the activated complexes to bind DNA decreased significantly after Sephadex G-50 chromatography (average decrease = $33\% \pm 4$, SD, $n = 3$). However, the initial level of DNA binding ability could be restored by the addition of either HeLa or HTC cell nuclear extract (Fig. 2). These data argue that a subpopulation of activated glucocorticoid receptor–steroid complexes from HeLa cells is dependent on the presence of a low molecular weight factor for DNA binding. Thus heterogeneity among activated glucocorticoid complexes is not unique for rat receptors but can also be seen with human receptors. Furthermore, factor(s) capable of increasing the DNA binding of one subpopulation of activated complexes is also found in non-rat cell lines.

Effect of sodium arsenite and MMTS on the DNA binding of activated HeLa cell glucocorticoid complexes

The sub-populations of activated glucocorticoid receptors from HTC cells can also be distinguished

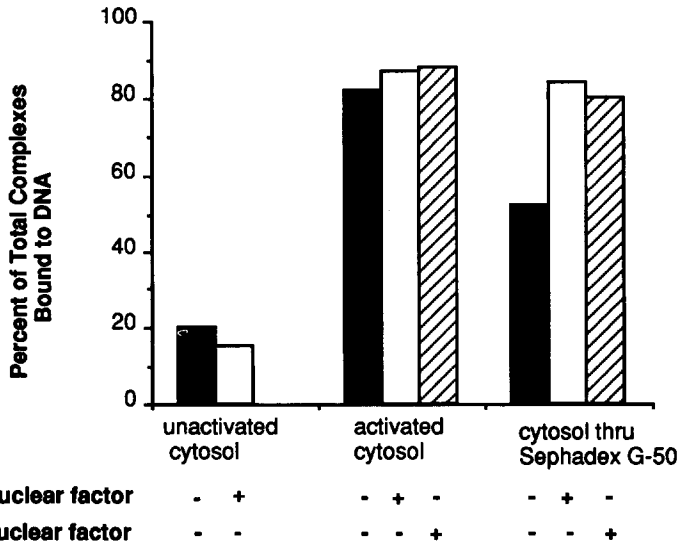

Fig. 2. DNA binding of HeLa cell glucocorticoid complexes. Glucocorticoid receptors were bound with [^{3}H]Dex, diluted with TAPS 8.8 buffer and either kept at 4°C (unactivated cytosol), or heated for 30 min at 20°C (activated cytosol), or heated (30 min/20°C) and then depleted of factor by gel filtration chromatography through Sephadex G-50. Complexes (40 μl) were incubated with either 80 μl of TAPS 8.8 or enriched HTC or HeLa cell nuclear factor for 20 min at 4°C. Samples were assayed on DNA/DEAE mini-columns and the extent of DNA binding determined as described in the legend for Fig. 1. Experiments with enriched HTC cell factor were performed once.

directly by their sensitivity to thiol reagents. The factor-dependent DNA binding of complexes is inhibited by sodium arsenite [11]. Sephadex G-50 chromatographed complexes that are devoid of factor are, as expected, refractory to sodium arsenite. Conversely, the factor-independent complex binding to DNA is inhibited by MMTS [11]. We therefore examined whether the sub-populations of activated human glucocorticoid complexes would display the same sensitivity to these thiol reagents. After activated HeLa cell complexes were incubated with 10 or 25 mM sodium arsenite, the DNA binding decreased to the same level as seen for cytosol which had been depleted of factor by Sephadex G-50 chromatography [Fig. 3(A)]. The addition of arsenite to factor-depleted cytosol did not cause any further decrease in DNA binding. Added factor regenerated the initial levels of DNA-binding activity in Sephadex G-50 chromatographed cytosol but not in the presence of sodium arsenite.

The addition of 1 mM MMTS to activated HeLa cell complexes caused a larger decrease in DNA binding [Fig. 3(B)]. When MMTS was added to factor-depleted cytosol, DNA binding was abolished. However, in this case, the DNA binding could be increased by the addition of factor. The extent of the increase was approximately equal to that seen when factor was added to the Sephadex G-50'd complexes. Thus MMTS inhibits the DNA binding of factor-independent, human glucocorticoid receptor–steroid complexes while arsenite blocks factor-dependent DNA binding.

DNA binding of activated progesterone receptor–steroid complexes

The above data demonstrated that both the heterogeneity of activated glucocorticoid complexes and the existence of a DNA-binding activity factor was neither tissue nor species specific. Therefore, the requirement of a similar factor for the maximal DNA binding of other receptors, such as activated progesterone receptor–steroid complexes from MCF-7 cells, was examined. As with glucocorticoid receptors, the addition of enriched MCF-7 cell nuclear factor had little effect on the DNA binding of unactivated progesterone receptor–steroid complexes (Fig. 4). However, unlike the preparations of glucocorticoid receptors (Fig. 2) [11, 32], occasional preparations of activated receptor–progesterone complexes responded to the addition of enriched nuclear factor(s) to display an increase in DNA binding even before removing any potential factors (c.f. Figs 4 vs 5). We suspect that such factor-induced increases in DNA binding of activated progesterone complexes reflects variations in amount of factor(s) in each cytosol preparation since the maximum amount of DNA-bound complexes was always ~70% (c.f. Fig. 5). For this reason, we usually added enriched nuclear factor(s) to activated cytosol to determine the maximal DNA binding of progesterone complexes. Interestingly, as shown in Fig. 4, nuclear extracts from either MCF-7 or HTC cells were equally effective in affording maximal binding even though HTC cells do not contain progesterone receptors [38] (data not shown).

Sephadex G-50 chromatography of activated progesterone complexes caused a decrease in the DNA-binding capacity of the complexes (average decrease from maximal binding = 59 ± 15%, SD, $n = 3$) which could be restored by the addition of enriched nuclear factor(s) from either MCF-7 or HTC cells (Fig. 4). The factor from MCF-7 cell nuclear extracts was found to have properties identical to those described for HTC

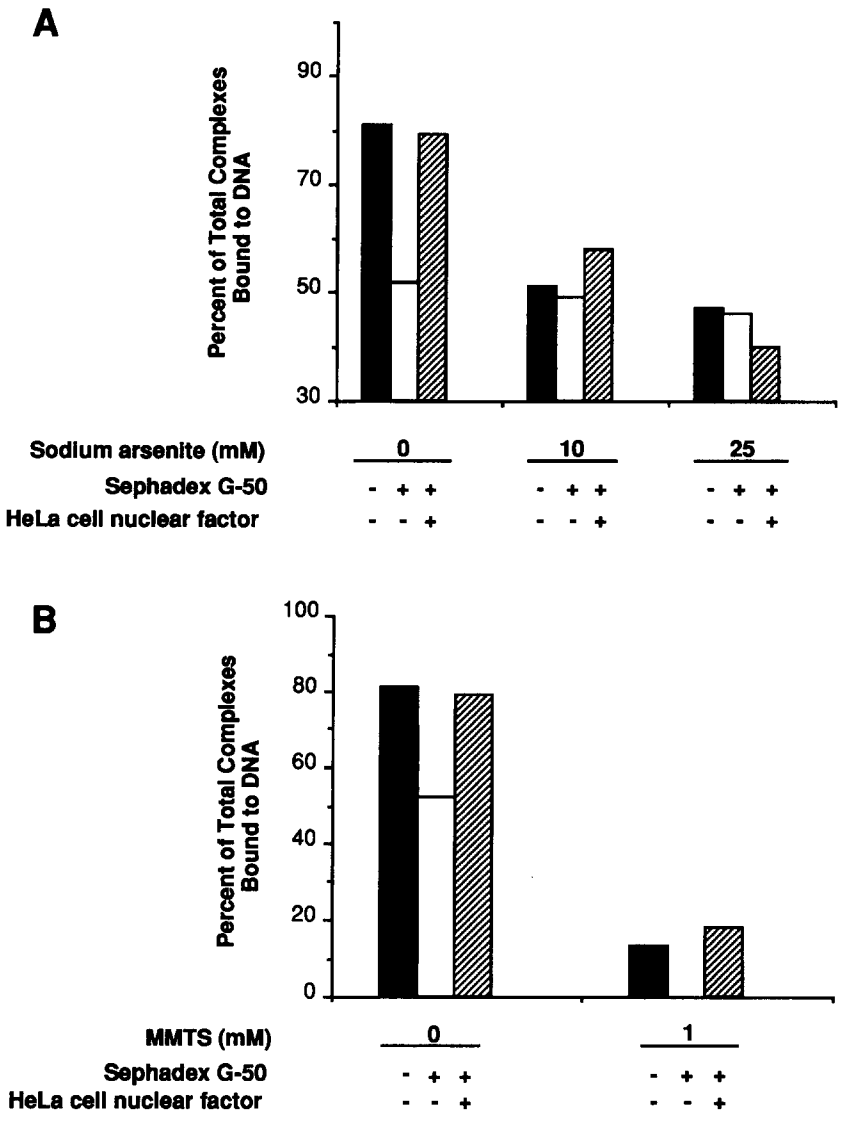

Fig. 3. Effect of sodium arsenite and MMTS on the DNA binding of glucocorticoid receptors from HeLa cells. Activated [^{3}H]Dex bound receptors (40 μl) were incubated with either 80 μl of TAPS 8.8 buffer or enriched nuclear factor plus 100 × preparations of either sodium arsenite (A) for 20 min at 0°C, or MMTS (B) for 30 min at 0°C, to achieve the final concentrations indicated. In those cases where the sample was chromatographed over Sephadex G-50, all additions occurred after the chromatography. The extent of DNA binding was assayed on DNA/DEAE mini-columns and quantitated as described in the legend for Fig. 1 after subtracting the background binding to cellulose columns.

cell nuclear factor [32], i.e. heat stable and an estimated M_w between 700–3000 (data not shown). Hence, activated progesterone receptor complexes are functionally heterogeneous and an apparently identical DNA-binding activity factor(s) is required for the maximal DNA binding of a sub-population of both activated progesterone and activated glucocorticoid receptors.

Effect of sodium arsenite and MMTS on DNA binding of progesterone complexes

We next examined whether the sub-populations of activated progesterone complexes could be distinguished by the same thiol reagents that selectively blocked the DNA binding of glucocorticoid receptor sub-populations. Thus, activated progesterone complexes, before and after being depleted of factor by chromatography on Sephadex G-50, were incubated with 25 mM sodium arsenite and then assayed for DNA-binding ability on DNA/DEAE mini-columns. Arsenite occasionally reduced the DNA binding to levels somewhat less than that seen after Sephadex G-50 chromatography (Fig. 5), presumably because not all of the factor had been removed by Sephadex G-50 chromatography. Such incomplete removal of factor had sometimes been observed for glucocorticoid complexes [11]. Support for this explanation comes from the fact that the combination of Sephadex G-50 chromatography and arsenite was not significantly more effective in reducing DNA binding than was arsenite alone (Fig. 5). As with glucocorticoid complexes [11], the addition of factor to arsenite-treated progesterone complexes had no effect on the level of

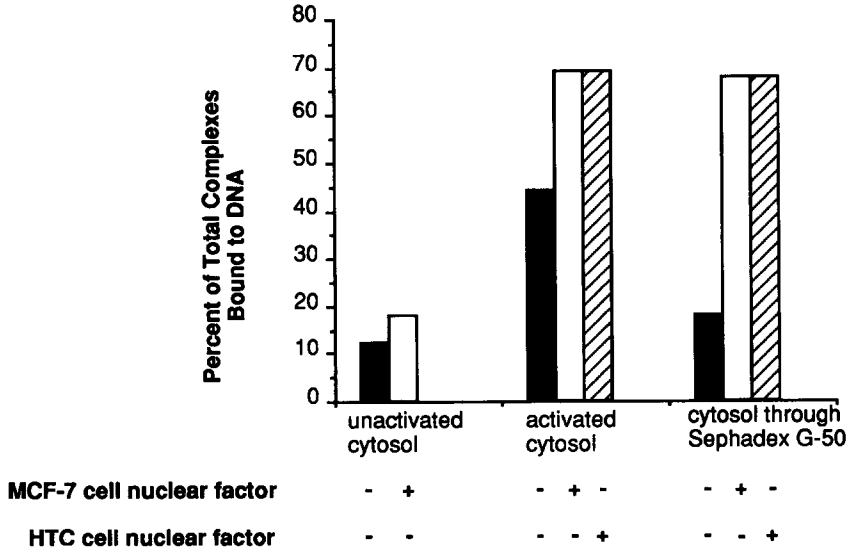

Fig. 4. Effect of factor on DNA binding of progesterone receptor–steroid complexes. Progesterone receptors bound with [^{3}H]R5020 were kept at 4°C (unactivated cytosol) for the duration of the experiment or activated by heating at 20°C with or without subsequent chromatography on Sephadex G-50. Aliquots of each cytosol (40 μl) were incubated with enriched factor (80 μl) from either MCF-7 or HTC nuclei and assayed on DNA/DEAE mini-columns. After subtracting the background binding to cellulose columns, the percentage of added complexes bound to DNA was determined as described for Fig. 1.

DNA binding. These effects could be reversed by the addition of dithiothreitol after removing the free arsenite (data not shown), consistent with results seen with glucocorticoid receptors [11, 32]. It should be noted that the addition of dithiothreitol itself had no effect on the DNA binding of progesterone receptors. In addition, nuclear extracts had no detectable thioredoxin reductase activity when assayed by the method of Holmgren [39] (data not shown).

MMTS eliminated most of the DNA binding of activated progesterone complexes (Table 1). The minimal binding that is retained may be a function of relatively low levels of factor in progesterone receptor preparations since the addition of factor to MMTS-treated, Sephadex G-50 chromatographed receptors always caused increased DNA binding. In fact, with HTC cell glucocorticoid receptors, the addition of factor to MMTS-treated, Sephadex G-50 chromatographed receptors also caused an increase in DNA binding [11]. Therefore, we can not yet say whether MMTS blocks the DNA binding of both sub-populations of progesterone receptors. The inclusion of this

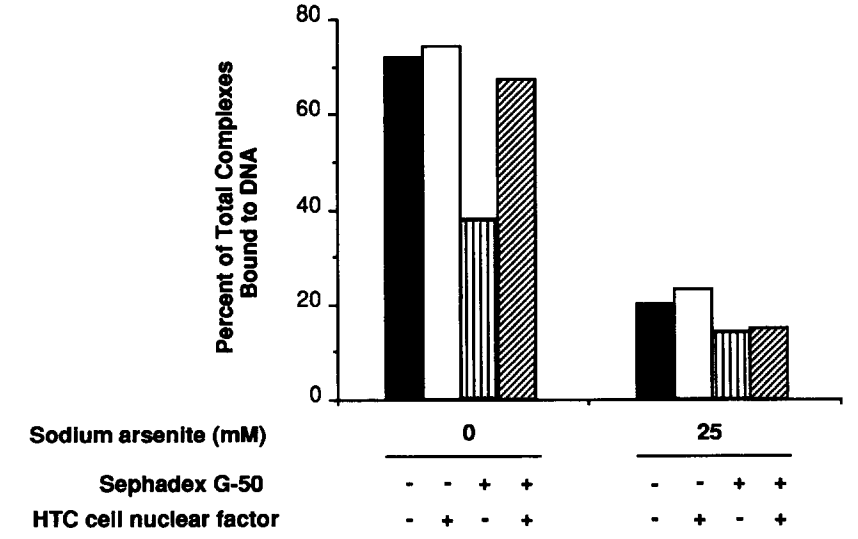

Fig. 5. Effect of sodium arsenite on the DNA binding of progesterone receptor–steroid complexes. Progesterone receptors were bound with [^{3}H]R5020, activated, and where indicated, depleted of factor by chromatography on Sephadex G-50. Aliquots (40 μl) of receptor-steroid complexes were then incubated with the indicated combinations of both sodium arsenite and/or 80 μl of enriched HTC cell nuclear factor. DNA binding was assayed by DNA/DEAE mini-columns After subtracting the background binding (see Experimental), the percentage of complexes binding to DNA were determined as described for Fig. 1.

Table 1. Effect of MMTS on progesterone receptor complex binding to DNA

Receptor source	% of added receptor–steroid complexes bound to DNA
Activated cytosol	42 (64)
+3 mM MMTS	2.3
Cytosol through Sephadex G-50	17 (36)
+MMTS	0
+Factor	41 (63)
+Factor and MMTS	7.5

Activated cytosol and cytosol depleted of factor by Sephadex G-50 chromatography were prepared as described in Experimental. The amount of added complexes was determined either by the dextran-coated charcoal assay [35] for activated cytosol or by direct counting in the case of Sephadex G-50'd cytosol. Aliquots of receptor–steroid complexes (40 μl) were mixed with 80 μl of enriched MCF-7 cell nuclear factor and assayed on DNA/DEAE mini-columns. A blank cellulose column was run to determine the background levels of complex binding. Because not all receptors bound to the mini-columns after MMTS treatment, the DNA binding is given as percent of added complexes. Thus after subtracting the background, the percentage of receptor binding to DNA was expressed as (dpm bound to DNA/dpm input) × 100. The data in parentheses are the normally expressed values (percent of total complexes bound to DNA) for non-MMTS-treated samples.

thiol reagent also caused an ~30% reduction in the recovery of progesterone complexes on the DNA/DEAE mini-columns, suggesting that the DEAE binding of one population of progesterone receptors is inhibited (data not shown).

A possible cause for the somewhat different DNA-binding properties of MMTS-treated progesterone and glucocorticoid complexes could be pH. The DNA binding of progesterone receptors was performed at a lower pH (7.6) than that of glucocorticoid receptors (pH 8.8). At the lower pH, the ionization of thiols would be decreased which could, in turn, effect reactions with MMTS and/or DNA binding. Activated progesterone complexes were therefore incubated with MMTS at the same pH (8.8) as used for glucocorticoid complexes. At this higher pH, MMTS still blocked the DNA binding of all, and the DEAE–cellulose binding of some, progesterone receptors (data not shown). Similarly, a preliminary experiment revealed no differences in the DNA binding properties of HTC cell glucocorticoid receptors at pH 8.8 and 7.6 (data not shown). Thus the dramatically decreased DNA binding of MMTS-treated progesterone was not a function of pH.

DNA binding of activated estrogen receptor–steroid complexes

Estrogen receptors were prepared from calf uterine tissue. Only 30% of the receptor–steroid complexes isolated under our conditions could be activated to a DNA-binding state when assayed on DNA/DEAE mini-columns (Fig. 6). While this amount of activation is low, it is not unusual for estrogen receptors [40, 41]. The addition of enriched HTC cell nuclear factor to activated estrogen receptor complexes did not significantly increase the DNA binding. Sephadex G-50 chromatography did, however, decrease the amount of DNA binding by 30 ± 4% (SD, $n = 4$) and the original level of binding could be restored by the addition of enriched HTC cell factor(s) (Fig. 6). Identical results were obtained when using either factor from calf uterine cytosol or, in one experiment, enriched calf uterine nuclear factor (data not shown). Therefore, a subpopulation of estrogen receptors also requires a small molecular weight factor(s) for DNA binding. In

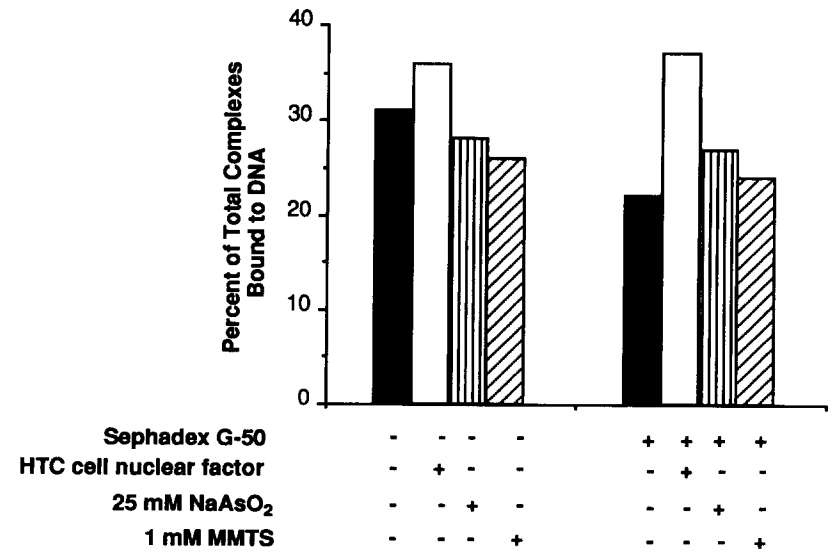

Fig. 6. Binding of estrogen receptor–steroid complexes from calf uterus to DNA. Activated, [^{3}H]estradiol-bound estrogen receptors and HTC cell nuclear extracts were prepared as described in Experimental. Activated complexes were incubated with sodium arsenite or MMTS or chromatographed on Sephadex G-50. Cytosol (40 μl) was incubated with enriched HTC cell nuclear factor (80 μl) and the mixture assayed on DNA/DEAE mini-columns. The percentage of DNA bound complexes was determined as described for Fig. 1.

Table 2. Binding of affinity labeled glucocorticoid complexes to DNA

Receptor source	DNA-bound complexes as % of total for receptors associated with	
	Dex-Mes	Dex
A		
Activated cytosol	72	64
Activated cytosol through Sephadex G-50	25	29
Sephadex G-50 cytosol + HTC cell nuclear factor	41	48
B		
Activated cytosol	55	
Activated cytosol through Sephadex G-50	30	
Activated cytosol + 3 mM MMTS	14	
Activated cytosol + 25 mM $NaAsO_2$ through Sephadex G-10	26	

(A) Glucocorticoid receptors from HTC cells were labeled with either [^{3}H]Dex-Mes or [^{3}H]Dex and activated by heat and dilution. An aliquot (0.5 ml) was depleted of factor by chromatography on Sephadex G-50 as described previously. The non-specific binding of [^{3}H]Dex was eliminated by Sephadex G-50 chromatography. Factor-depleted cytosol (400 μl) was incubated with 800 μl of enriched HTC nuclear extract or TAPS 8.8 buffer and chromatographed on 3 ml DNA–cellulose columns. Receptor–steroid complexes bound to DNA were eluted as described in Experimental. Specifically bound [^{3}H]Dex complexes were determined directly by counting both the starting receptor solutions and the various fractions. For [^{3}H]Dex-Mes labeled receptors, both the starting receptor solutions and those eluted from the column were analyzed on 10.8% SDS-polyacrylamide gels as described previously [34]. The specifically labeled material in the region of 98 kDa was determined by slice and counting followed by subtracting the non-specific labeling, as determined from the dpm in the adjacent slices. For both [^{3}H]Dex bound and [^{3}H]Dex-Mes labeled receptors, the complexes specifically bound to DNA were expressed as percent of total observed complexes{ = [complexes bound to DNA/(complexes bound to DNA + complexes recovered in flow thru)] × 100}. (B) [^{3}H]Dex-Mes labeled activated cytosol was either depleted of factor by chromatography on Sephadex G-50 or incubated with MMTS or sodium arsenite at the concentrations indicated for 20 min at 4°C. The samples containing arsenite were then chromatographed on Sephadex G-10 to remove free arsenite. All samples were then chromatographed on 3 ml DNA–cellulose columns and the percentage of recovered complexes that bound to DNA was quantitated as in A.

contrast to the behavior seen with progesterone and glucocorticoid receptors, the addition of sodium arsenite or MMTS to activated estrogen receptor complexes had little effect on the subsequent DNA binding.

Binding of Dex-Mes labeled receptors to DNA

Having found evidence for functional heterogeneity among activated glucocorticoid, progesterone and estrogen receptors bound with agonists, we next inquired whether functional heterogeneity could also be observed among antagonist-bound receptors. For these studies, we chose HTC cell glucocorticoid receptors that had been covalently labeled with the antiglucocorticoid Dex-Mes [42, 43] and found, by fluorography, to be intact 98 kDa receptors (data not shown). Since Dex-Mes labels many proteins non-specifically, the DNA binding of [^{3}H]Dex-Mes labeled receptor–steroid complexes could not be analyzed on DNA/DEAE mini-columns. Instead, receptor–steroid complexes were eluted from 3 ml DNA–cellulose columns and quantitated by slice-and-counting after gel electrophoresis on denaturing gels. Chromatography of Dex-Mes-labeled complexes on Sephadex G-50 columns decreased the amount of DNA binding [Table 2(A)] just as had been seen with Dex-bound complexes [32]. The addition of enriched HTC nuclear factor(s) increased the DNA binding of the factor-depleted cytosol. The amount of reconstitution was not as complete as that seen on mini-columns with Dex-bound receptors (Figs 2 and 3) [32]. However, this apparent decrease in reconstitution was found to be an artifact of the DNA–cellulose column vs the mini-column assay. If Dex-bound complexes are analyzed in the same manner, then the degree of reconstitution was similarly reduced [Table 2(A)]. We therefore conclude that Sephadex G-50 chromatography and nuclear factor affect the DNA binding of Dex-bound and Dex-Mes-labeled complexes equally.

The amount of Dex-Mes labeled receptors capable of binding to DNA decreased after treatment with arsenite to values approaching that after Sephadex G-50 chromatography (Table 2B). As seen with Dex-bound glucocorticoid complexes, treatment of the Dex-Mes-labeled receptors with MMTS caused an even greater reduction in the DNA binding. Hence it was concluded that antagonist-labeled complexes exhibited the same factor-dependent functional heterogeneity

Table 3. Binding of agonist and antagonist receptor–steroid complexes to HTC cell nuclei

Receptor source	% of added complexes bound to nuclei for receptors associated with Dex	Dex-Mes
Activated cytosol		
Crude nuclei	47	16
Washed nuclei	45	15
Activated cytosol through Sephadex G-50		
Crude nuclei	52	16
Washed nuclei	50	20

Glucocorticoid receptors from HTC cell cytosol were either bound with [^{3}H]Dex (agonist) or labeled with [^{3}H]Dex-Mes (antagonist) and incubated with nuclei isolated from HTC cells as described previously [32]. Briefly, crude nuclei were prepared from rupturing HTC cells by freezing in liquid nitrogen. Where indicated, the pellet resulting from this step was washed with TAPS 8.8 buffer to generate washed nuclei. Receptor–steroid complexes were incubated with nuclei for 2 h at 0°C and the nuclear pellet washed with TAPS 8.8 buffer and extracted with 0.3 M NaSCN. Dex-bound complexes were determined by direct counting while Dex-Mes-labeled receptors were precipitated with TCA and analyzed by SDS-gel electrophoresis. The nuclear bound complexes are expressed as percentage of added complexes.

and sensitivity to thiol reagents as did the agonist-bound complexes.

Effect of factor on agonist- and antagonist-complex binding to isolated nuclei

We have previously reported that nuclear factor does not affect the nuclear binding of complexes [32] and that antagonist-labeled complexes bound less extensively to the nuclei of whole cells than did agonist bound complexes [44, 45] even though both complexes bound equally well to non-specific [35] and specific DNA sequences [46]. Thus the presence of receptor heterogeneity and the equal DNA binding of agonist and antagonist complexes could be an artifact of cell-free activation conditions. However, when agonist and antagonist complexes were compared for their ability to bind nuclei in a cell-free system, these differences were maintained (Table 3). In addition, when factor was removed from the cytosol by Sephadex G-50 chromatography, there was no change in the relative ability of the antagonist labeled complexes to bind either crude or washed nuclei.

Two-dimensional electrophoresis of sub-populations of activated glucocorticoid complexes

Charge heterogeneity among glucocorticoid receptors is a well established but poorly understood phenomenon [9, 47–49]. In order to determine if the above functional heterogeneity among activated glucocorticoid complexes is correlated with the different receptor isoforms, we analyzed both factor-dependent and -independent activated [^{3}H]Dex-Mes labeled glucocorticoid complexes by two dimensional gel electrophoresis. The factor-dependent and -independent sub-populations were isolated from DNA-cellulose columns after pre-treatment with MMTS or sodium arsenite, respectively. DNA-bound complexes from untreated activated cytosol contained both populations. Up to 5 separate isoforms of [^{3}H]Dex-Mes-labeled receptor could be resolved by 2-D gels at pIs from 6.1 to 6.5 (Fig. 7). However, no differences in relative abundance of each isoform could be detected within each sub-population of receptors.

Effect of factor on DNA binding of truncated receptor–steroid complexes

Although no physicochemical difference could be detected between those complexes that did or did not require factor for DNA binding, we previously reported that the DNA-binding activity factor did not bind to DNA but appeared to associate directly with activated glucocorticoid complexes [11]. Furthermore, preliminary data indicated no interaction between the factor and heparin columns, sodium arsenite, or MMTS (data not shown). Therefore, we attempted to define that region of the glucocorticoid receptor that was required for the heterogeneity in DNA binding properties. Full length (98 kDa) [^{3}H]Dex-bound glucocorticoid receptors were digested with chymotrypsin to generate the 42 kDa amino-terminal truncated receptor. Activated 98 and 42 kDa complexes bound to DNA equally (Table 4). Incubation of the 42 kDa complexes with sodium arsenite and MMTS showed the same heterogeneity of receptors as for intact 98 kDa receptor–steroid complexes. In addition, Sephadex G-50 chromatography of 42 kDa complexes caused an identical decrease in DNA binding which could be restored by the addition of factor (Table 4). Therefore we conclude that both the factor and the thiol reagents interact with amino acids in the 42 kDa receptor.

DISCUSSION

The previously demonstrated functional heterogeneity of activated receptor–glucocorticoid complexes, and the presence of a low molecular weight nuclear factor(s) required for the DNA binding of a sub-population of complexes, is now found not to be limited to HTC cell glucocorticoid receptors. An apparently identical DNA binding activity factor(s) was found in the nuclear extracts of rat liver and kidney. Activated glucocorticoid receptors from the human HeLa cell line also existed as a heterogeneous population in that the DNA binding of one sub-population required the presence of a small molecular weight factor(s) from HeLa cell nuclei, As with glucocorticoid receptors from HTC cells, the different receptor populations in HeLa cells could be distinguished by their sensitivity to the sulfhydryl reagents, sodium arsenite and MMTS. Interestingly, the factor-mediated DNA binding of activated HeLa cell complexes was neither tissue nor species specific since the factor in HTC cell nuclear extracts reconstituted the DNA binding of HeLa cell complexes just as efficiently as did the endogenous factor.

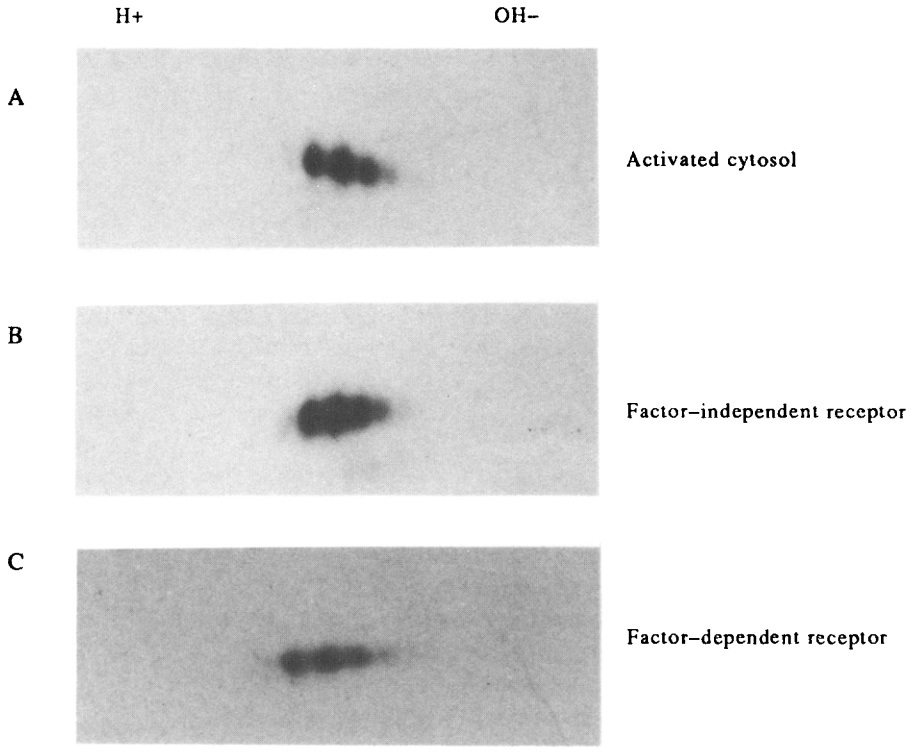

Fig. 7. Fluorograph of two-dimensional gel electrophoresis of factor-dependent and independent glucocorticoid receptor–steroid complexes from HTC cells. Receptors were labeled with [^{3}H]Dex-Mes and activated as described previously. [34]. Aliquots of activated cytosol were incubated with TAPS 8.8 buffer ± 25 mM sodium arsenite or 1 mM MMTS and chromatographed on 3 ml DNA-cellulose columns. The DNA-bound complexes were eluted with NaCl, desalted on mini Sephadex G-10 columns, and subjected to NEPHGE in the first dimension and SDS-polyacrylamide electrophoresis in the second dimension. (A) Dex-Mes labeled receptor bound to DNA–cellulose from activated cytosol (=both populations). (B) Dex-Mes labeled receptor bound to DNA after incubation with sodium arsenite (=factor-independent complexes). (C) Dex-Mes labeled receptor bound to DNA after incubation with MMTS (=factor-dependent complexes). The positioning of each panel was achieved by aligning the acidic end of each NEPHGE gel in the SDS-polyacrylamide gel. Control experiments under identical conditions with receptor, labeled by [^{3}H]Dex-Mes + non-radioactive Dex gave no signals (data not shown), thus establishing that the above material is receptor protein.

Factor-mediated DNA binding of a sub-population of receptors is not unique to glucocorticoid receptor complexes but has now been observed with other steroid receptors. After chromatography on Sephadex G-50, the DNA binding capacity of activated progesterone complexes was reduced but could be restored by the addition of a nuclear extract from either MCF-7 cells or HTC cells. Hence the DNA-binding activity factor(s) for both progesterone and glucocorticoid receptor complexes is neither species nor tissue specific. The reduction in DNA binding after Sephadex G-50 chromatography was somewhat greater for progesterone (59%) than glucocorticoid (33% for HeLa cell and 43% for HTC cell) receptors. This indicates that a higher percentage of progesterone receptors are factor dependent. As with glucocorticoid complexes, arsenite was found to block the DNA binding of factor-dependent activated progesterone complexes. The effect of MMTS on progesterone receptors may be different from that of glucocorticoid receptors in that MMTS appeared both to be more effective in blocking receptor binding to DNA and to inhibit the binding of one population of complexes to DEAE–cellulose.

A DNA-binding activity factor was also required for some estrogen receptor complexes to bind to DNA. As with the glucocorticoid and progesterone complexes, this required factor could be removed simply by Sephadex G-50 chromatography. The endogenous factor(s) for calf uterine estrogen complexes appeared identical to, and could be replaced by the factor(s) from HTC cells. Estrogen receptor complexes did not show functional heterogeneity when incubated with thiol reagents. Given the different distribution of cysteines in estrogen, glucocorticoid and progesterone receptors [50, 51] and the ability of sodium arsenite to block the steroid binding of only glucocorticoid receptors [52], the different responses of these receptors to thiol reagents was not entirely unexpected.

Finally, the existence of functional sub-populations of activated receptors is not limited to agonist complexes but was also seen with an antiglucocorticoid complex. Some receptor–antagonist complexes have been reported to have reduced DNA binding [53, 54]. However, we found that sub-populations of antagonist (Dex-Mes-labeled) and agonist (Dex-bound) glucocorticoid complexes are equally affected by factor in their DNA binding. This equivalent DNA binding was a puzzle in view of the well-known decreased nuclear

Table 4. DNA binding of 98 kDa and 42 kDa glucocorticoid receptor

Receptor source	% of total complexes bound to DNA	
	Expt. A	Expt. B
98 kDa		
Activated cytosol	80	82
+25 mM $NaAsO_2$	53	
+1 mM MMTS	18	
Cytosol through Sephadex G-50		64
+HTC cell nuclear factor		79
42 kDa		
Activated cytosol	86	86
+25 mM $NaAsO_2$	67	
+1 mM MMTS	19	
Cytosol through Sephadex G-50		67
+HTC cell nuclear factor		87

Glucocorticoid receptors from HTC cells were labeled with [^{3}H]Dex, activated, and digested with chymotrypsin (4 μg/ml) for 1 h at 4°C. The efficiency of digestion was found to be ~100%, as shown by the formation of 42 kDa [^{3}H]Dex-Mes-labeled complexes in parallel incubations (data not shown). Proteolysis was stopped by the addition of chymostatin (40 μg/ml); and, either sodium arsenite or MMTS was added to the indicated final concentration. Enriched factor was prepared from nuclear extracts of HTC cells as described in Experimental. Samples were incubated an additional 20 min and the extent of DNA binding determined by the DNA/DEAE mini-column procedure as described in the legend to Fig. 1.

binding in intact cells of Dex-Mes-labeled complexes relative to Dex-bound complexes [44, 45]. This, in turn, raised the question of whether the unequal binding behavior of Dex-Mes-labeled complexes was simply a reflection of different activation conditions in intact cells and in cell-free systems. Our ability to obtain decreased nuclear binding with cell-free activated, Dex-Mes-labeled complexes (Table 3) argues that the reduced nuclear binding is a function of the acceptor (e.g. chromatin vs DNA). These results also show that cell-free and whole cell activation conditions can give functionally identical complexes. This is significant because it argues that our observations of functional receptor heterogeneity and of a nuclear factor(s) required for the DNA binding of one sub-population are not an artifact of the cell-free conditions.

Given the fact that an apparently common factor(s) is required for the DNA binding of a sub-population of the activated complexes of three different steroid receptors and of an antisteroid complex, it seems reasonable to propose that such a low M_w factor(s) may be involved in the DNA binding of a sub-population of all receptor–steroid complexes. Numerous other studies have documented the involvement of larger factors ($M_w > 5$ kDa) in the DNA binding of some steroid receptors [6, 7, 23, 29–31] and of other members of the superfamily of steroid/thyroid receptors [24–28]. However, in many of these reports, especially those employing gel shift assays, no quantitation of the affected receptor–steroid complexes was possible because only a small fraction of the added complexes bound to DNA. As far as we are aware, our low M_w factor(s) is the first to be shown to affect a distinct sub-population of receptors. This result is not incompatible with previous observations but rather nicely explains the low amounts of DNA binding that are often reported for partially purified complexes which would lack low M_w factors (see [32] and references therein). Further studies are required to determine whether the larger M_w factors will also be found to have selective actions on receptor sub-populations. The selective action of at least our low M_w factor(s) also raises interesting possibilities for additional control of steroid regulated gene transcription.

An obvious question concerning the biological relevance of our low M_w factor(s) is whether the effects of factor on receptor sub-populations is limited to the binding to non-specific DNA or are also seen for the specific binding to the hormone response elements shown to be involved in receptor–steroid complex regulation of responsive genes. Most of the larger factors do increase the binding to the hormone response elements in gel shift assays. Thus these factors could be involved in the action of hormone regulated gene transcription. It is, therefore, of interest that the factor involved in the DNA binding of thyroid receptors is not active for all thyroid responsive elements [55, 56]. We are currently examining the effect of our low molecular weight factor on receptor binding to glucocorticoid responsive elements but not by gel shift assays because of the presence of large amounts of a non-receptor species which comigrate with the specific DNA–receptor complex (Cavanaugh and Simons, unpublished results). However, even if there is no effect of factor on specific DNA binding, the importance of factor-regulated receptor–steroid complex binding to non-specific DNA sequences cannot be ignored for several reasons. The binding kinetics of *trans*-acting factors to specific sequences depends crucially on non-specific DNA [57, 58]. In this respect, it should be noted that intersegment transfer of binding proteins, which is thought to be of major importance in locating the specific DNA binding sites, may be greatly facilitated by dimeric forms of DNA-bound proteins, such as are observed for steroid receptors [59, 60]. In addition, without non-specific DNA binding to act as a buffer, activated complex binding to a small number of high-affinity steroid responsive elements would become saturated, resulting in full gene activation, over a very narrow concentration of steroid [61], thus leaving little room for regulation.

In an attempt to identify that region of the glucocorticoid receptor which is required for the expression of functional heterogeneity, we examined the 42 kDa chymotrypsin fragment containing the DNA and steroid-binding domains. The DNA binding of this 42 kDa fragment was still responsive to factor preparations and thiol reagents. Further experiments are required to determine whether or not the sensitive sequences lie within the steroid binding domain, which is already

crowded with other activities [62]. Domain swap experiments [63] with the estrogen receptor would be useful in localizing those regions which interact with factor and/or thiol reagents.

One tempting link between the functional and physicochemical heterogeneity of glucocorticoid receptors has been their charge heterogeneity. Smith *et al.* [64] reported that chromatography on DNA–cellulose eliminated the more acidic of the two receptor isoforms that they had resolved by two-dimensional electrophoresis of activated complexes. In contrast, we observed five isoforms of Dex-Mes-labeled receptor (Fig. 7), in closer agreement with data reported by others [48]. More significantly, however, in our hands, DNA–cellulose chromatography did not appear to eliminate the more acidic forms (Fig. 7) nor could we discern any differences in isoform composition between the factor-dependent and -independent receptor subpopulations. It cannot be ruled out that electrically neutral modifications of amino acids are responsible for functional heterogeneity or that different amino acids are similarly modified to yield the same net charge in either population.

In conclusion, the existence of functional heterogeneity among activated estrogen, glucocorticoid and progesterone receptors is not limited to complexes of agonist steroids. This, plus the widespread occurrence of a small molecular weight factor(s) that is required for the DNA binding of one sub-population of activated complexes, suggests that the same phenomenon may apply for all of the steroid receptors. If the level or activity of the low M_w factor(s) can be modified in the cell, this would offer yet another level of control for steroid regulated gene expression [65]. Further studies are required to test this intriguing hypothesis.

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