Biochemical Pharmacology, Vol. 45, No. 5, pp. 1121–1128, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

BINDING OF THE Ah RECEPTOR TO RECEPTOR BINDING FACTORS IN CHROMATIN

ROBERT T. DUNN, II, THOMAS S. RUH and MARY F. RUH*
Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, St. Louis, MO 63104, U.S.A.

(*Received* 17 *June* 1992; *accepted* 16 *November* 1992)

Abstract—Dioxin induces biological responses through interaction with a specific intracellular receptor, the Ah receptor, and the subsequent interaction of the Ah receptor with chromatin. We report the binding of the Ah receptor, partially purified from rabbit liver, to receptor binding factors in chromatin. Rabbit liver chromatin proteins (CP) were isolated by adsorption of chromatin to hydroxylapatite followed by sequential extraction with 1–8 M GdnHCl. To assay for receptor binding a portion of each CP fraction was reconstituted to rabbit double-stranded DNA using a reverse gradient dialysis of 7.5 to 0 M GdnHCl. These reconstituted nucleoacidic proteins were then examined for binding to [^{3}H]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin ([^{3}H]TCDD)-receptor complexes by the streptomycin filter assay. Prior to the binding assay, [^{3}H]TCDD-receptor complexes were partially purified by step elution from DEAE-cellulose columns. CP fractions 2, 5, and 7 were found to bind to the Ah receptor with high affinity. Scatchard analysis yielded K_d values in the nanomolar range. Competition with 2-fold excess unlabeled TCDD-receptor complexes was demonstrated, and binding was reduced markedly when the receptor was prepared in the presence of 10 mM molybdate. Such chromatin receptor binding factors (RBFs) may participate in the interaction of receptor with specific DNA sequences resulting in modulation of specific gene expression.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)† is a well studied representative of a group of halogenated aromatic hydrocarbons that have caused a great deal of concern as environmental contaminants. TCDD (dioxin) produces a large variety of species and tissue effects as well as demonstrating a sex preference in some species [reviewed in Refs. 1 and 2]. The biological actions of dioxin are believed to occur by initially binding to the Ah (aryl hydrocarbon) receptor [3–5] and causing the receptor to acquire the ability to bind DNA tightly [6, 7], especially the dioxin response element [8, 9], and thus modifying gene transcription [10]. The Ah receptor appears to form a heterodimer with a non-TCDD binding protein [11, 12] which may be important for the interaction of the receptor complex with DNA [11].

These series of events resemble the events in steroid hormone action. Although the Ah receptor has been shown recently to be a member of the group of basic region/helix-loop-helix (BR/HLH) DNA binding factors [13], the mechanisms nonetheless are similar in terms of a receptor which binds ligand, undergoes a transformation, and binds specific sequences of DNA. These similarities provide insight into the study of the Ah receptor and its mechanism of action. Models of dioxin action must take into account the range of responses that dioxin produces as well as explain the toxicity differences among species. There is a correlation between potency to induce the enzyme aryl hydrocarbon hydroxylase (AHH) and toxic potency. However, it has also been suggested that toxicity may be related to long-term effects (i.e. epithelial hyperplasia) requiring persistent receptor occupancy [1].

Both our laboratory [14, 15] and other investigators [16, 17] have postulated that specific non-histone chromosomal proteins are candidate components of acceptor sites for steroid hormone receptors which confer high affinity and specific binding to DNA. Therefore we sought to determine if such chromatin receptor binding factors exist for the Ah receptor (AhRBFs). Such studies would allow further comparison of the mechanism of action of the Ah receptor with that of steroid hormone receptors.

The rabbit has been shown to be a species sensitive to the effects of halogenated aromatic hydrocarbons (HAHs) [18]. Livers of rabbits dermally exposed to HAHs exhibited lesions as well as increases in relative and absolute weight which were associated with the exposure. Since our future studies will involve further purification of RBFs for the Ah receptor, rabbit liver was the tissue of choice since substantial amounts of easily obtainable tissue will be required.

MATERIALS AND METHODS

Chemicals and buffers. [^{3}H]TCDD (40 Ci/mmol) was obtained from Cambridge Isotopes, Cambridge,

* Corresponding author: Dr. Mary F. Ruh, Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, 1402 South Grand Blvd., St. Louis, MO 63104. Tel. (314) 577-8551; FAX (314) 577-8554.

† Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; AhRBF, Ah receptor binding factor; HAP, hydroxylapatite; CP, chromosomal protein; dsDNA, double-stranded DNA; and NAP, nucleoacidic protein.

MA, and was repurified prior to use by HPLC. DEAE cellulose was obtained from Whatman, Clifton, NJ. Ultrapure guanidine hydrochloride (GdnHCl) was obtained from Amresco, Solon, OH. Hydroxylapatite (HAP) was obtained from Bio-Rad, Richmond, CA. All other chemicals were analytical grade. TESH/GP buffer was 10 mM Tris–HCl, 1 mM EDTA, 5% glycerol (v/v), 0.5 mM phenylmethylsulfonyl fluoride, 0.11% monothioglycerol (v/v), pH 7.5, at 4°. Reconstitution buffer was 0.01 M EDTA, 0.05 M sodium acetate, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, pH 6.0.

Preparation of chromatin and DNA. The isolation of chromatin from rabbit liver was as previously described [14] except that Triton X-100 was omitted. The preparation of rabbit spleen DNA [19] was as previously described.

Isolation and purification of the chromosomal proteins (CP). In general, the isolation of chromosomal proteins was as previously described [20]. Chromatin was prepared in quantities sufficient for initial detection and characterization of RBF activity. HAP was hydrated in 170 mL of 3 M NaCl in 100 mM potassium phosphate buffer, pH 6, for a minimum of 12 hr and chromatin previously isolated from 186 g rabbit liver (Pel-Freez Biologicals, Rogers, AR) was added (67.5 mg DNA per 34 g HAP) and stirred every 15 min with a glass rod at 4° for 2 hr. The histones were removed by filtration. A second 2-hr extraction with 170 mL of 3 M NaCl was performed which was filtered immediately after mixing with the HAP-chromatin. Then increasing molarities (1–8 M) of GdnHCl in 100 mM potassium phosphate buffer were used to extract the non-histone proteins. Each incubation with the HAP-chromatin slurry for each molarity of GdnHCl (1–8 M) was for 50 min and was followed by filtration to provide each chromosomal protein fraction, resulting in 8 fractions of protein (CP 1–8). The 125-mL fractions were then dialyzed against 10 vol. of distilled water with six changes over 48 hr followed by dialysis in 5% acetonitrile in water for 1 hr. The samples were lyophilized and stored desiccated at room temperature until further use.

Preparation and partial purification of Ah receptor complexes. Livers from young rabbits were received frozen on solid CO_2 from Pel-Freez Biologicals and stored at −80°. Liver was thawed, minced, and homogenized in 5 vol. (v/v) TESH/GP buffer using a Kontes No. 24 glass/glass homogenizer. The homogenate was then centrifuged at 105,000 *g* for 30 min (Type 70.1 Ti rotor, Beckman) to obtain the cytosol fraction. All steps were performed at 0–4°.

Cytosol was incubated with 10 nM [^{3}H]TCDD, in a maximum volume of 10 μL of 1,4-dioxane vehicle per 1 mL cytosol, for 90 min at 4°. After incubation, unbound ligand was adsorbed by dextran-coated charcoal. The cytosol (20 mL) was then loaded onto a pre-equilibrated DEAE-cellulose column (packed volume 11 mL). After washing the column with 40 mL TESH/GP buffer containing 0.07 M KCl, Ah receptor was eluted by step elution using 0.35 M KCl. Fractions (3 mL) were collected, and aliquots (50 μL) were counted in 4 mL of Ready-Protein scintillation fluid (Beckman) at 47.5% efficiency using a Beckman LS-7500 scintillation counter. The peak fraction containing Ah receptor was used in the cell-free binding assay.

Sucrose density analysis of Ah receptor preparation. Linear 5–20% sucrose density gradients (3.6 mL) containing either 0.3 or 0.15 M KCl in TESH/GP buffer were prepared and chilled at 4°. An aliquot of the peak fraction of partially purified [^{3}H]TCDD-receptor complexes, eluted in 0.3 M KCl, was placed on a sucrose density gradient prepared in 0.3 M KCl. Similarly, an aliquot from the same peak fraction but with the salt concentration adjusted to 0.15 M KCl was placed on a sucrose density gradient prepared in 0.15 M KCl. The gradients were then centrifuged (225,000 *g* 16 hr at 5°) in an L5-50 ultracentrifuge (Beckman) using the SW-56 swinging bucket rotor. Fractions (4 drops) were collected from the top and counted in 4 mL of Ready-Protein scintillation fluid (Beckman). To determine that the sucrose density gradient peaks were specific for Ah receptor, cytosol was prepared and incubated with 10 nM [^{3}H]TCDD + 200-fold excess 2,3,7,8-tetrachlorodibenzofuran (TCDF). After DEAE-column chromatography, sucrose density gradient analysis was performed as above. [^{14}C]Ovalbumin (3.7 S) and [^{14}C]γ-globulin (6.6 S) were added either as internal standards or in parallel gradient tubes.

Cell-free receptor binding assay. Ah receptor binding to reconstituted RBFs was measured using a modification of the method of Spelsberg [21]. Briefly, isolated chromatin protein fractions in 7.5 M GdnHCl were reconstituted in 1-mL dialysis bags to rabbit double-stranded DNA (dsDNA) (0.5 mg) at a 1 : 10 ratio of protein : DNA using a reverse gradient dialysis procedure which reduced 7.5 M GdnHCl to 0.0 M GdnHCl over a 24-hr period. This procedure produced a reconstituted nucleoacidic protein (NAP) preparation. The NAP solutions were centrifuged for 20 hr at 110,000 *g* and the pellets dissolved in 1.2 mL of 10 mM KCl in 2 mM Tris, 0.1 mM EDTA, pH 7.5. To test for receptor binding, DEAE-cellulose partially purified [^{3}H]TCDD-receptor (0.3 pmol) was added to approximately 70 μg NAP as DNA in a final assay volume of 500 μL (0.15 M KCl). After incubation on ice for 1 hr with frequent vortexing, 100 μL of 1.2% streptomycin sulfate solution was added to precipitate the receptor-NAP complexes and incubation was continued for 30 min. The assay tubes were centrifuged at 800 *g* for 10 min and the supernatant was aspirated. The pellets were washed two times with 1 mL of 0.02% streptomycin sulfate solution, then suspended in the same solution and decanted onto Millipore (24 mm type HA) filters. The filters were washed three times with 1.0 mL of 0.02% streptomycin sulfate solution, air dried for 15 min, and then dried under an infrared lamp for 15 min; the radioactivity on the filters was counted in 10 mL of scintillation fluid (0.4% Omnifluor-xylene) and then the filters were removed and dried, and the DNA content was determined by the method of Burton [22]. Binding to DNA alone was subtracted from all values and data are presented as picomoles of bound [^{3}H]TCDD-receptor complexes per milligram of DNA.

Saturation and Scatchard analysis. Freshly prepared cytosol was incubated with 10 nM [^{3}H]TCDD

and subjected to DEAE-cellulose chromatography to partially purify the Ah receptor as above. Various aliquots (25–250 μL) of the receptor preparation were added in triplicate to the cell-free binding assay as outlined above. Dissociation constants (K_d) and the number of binding sites per cell (B_{max}) were calculated using Scatchard analysis [23].

Specificity experiments. Cytosol was incubated with 10 nM [^{3}H]TCDD or 10 nM unlabeled TCDD and subjected to DEAE-cellulose chromatography to partially purify the Ah receptor as above. Also prepared was cytosol which had been heated at 34° for 75 min and partially purified to obtain a denatured Ah receptor (dR) preparation. The denatured receptor preparation was examined for binding by [^{3}H]TCDD and no binding was measurable. Binding of [^{3}H]TCDD-receptor complexes to NAPs 2, 5, and 7 was performed in the presence of [^{3}H]TCDD-receptor + 2-fold excess denatured receptor (to keep protein concentration constant) for total binding, or in the presence of [^{3}H]TCDD-receptor plus 2-fold excess TCDD-receptor for non-specific binding. The TCDD-receptor preparation was exactly as described for the [^{3}H]TCDD-receptor complex, except that a portion of the same cytosol preparation was incubated with 10 nM unlabeled TCDD. The same fraction of DEAE-purified receptor was used for both preparations. Such a protocol should result in the same concentration of ligand-bound Ah receptors in the binding assay. Experiments were also performed to measure binding to DNA alone.

Preparation of Ah receptor in the presence of 10 mM molybdate. Ah receptor was prepared as above except that 10 mM molybdate was present in the homogenization buffer as well as in all solutions in the partial purification of the Ah receptor to attempt to keep Ah receptor in the non-transformed state.

RESULTS

Partial purification of [^{3}H]TCDD-receptor complexes. In the present study we partially purified Ah receptor complexes from rabbit liver cytosol using DEAE-cellulose ion exchange column chromatography. The receptor was eluted from the column using a KCl step elution procedure generating a 30-fold partially purified, salt transformed, Ah receptor complex. Since the peak fraction elutes at 0.3 M KCl and the binding assay is performed at 0.15 M KCl, we analyzed the receptor form under these conditions using sucrose density gradient analysis. A 200-μL aliquot of the peak fraction eluted in 0.3 M KCl was layered on a 5–20% sucrose density gradient prepared in 0.3 M KCl. Under these conditions the Ah receptor sedimented in monomeric form at 4 S (Fig. 1A). Upon adjustment of the receptor preparation to 0.15 M KCl, a 200-μL aliquot was layered on a 5–20% sucrose density gradient prepared in 0.15 M KCl. The receptor sedimented in dimeric form at 6 S under these conditions (Fig. 1A). This is the form of the receptor used in the binding assay. Others have shown that the 6 S form is the DNA binding form of the Ah receptor *in vivo* [24–26]. It has also been shown that nuclear salt-extractable Ah receptors from a variety of species sediment at 6 S [27, 28].

That the peak observed in the sucrose density gradient experiments is specific for Ah receptor was shown by competition with TCDF. A 200-fold excess of TCDF added to the cytosol incubation displaced the [^{3}H]TCDD from the Ah receptor in the DEAE-purified fraction, thereby reducing the receptor peak (Fig. 1B). From these experiments as well as from determinations of non-specific binding of [^{3}H]TCDD to preparations containing heat-denatured Ah receptors, the non-specific binding of [^{3}H]TCDD was estimated to be 40–50% in the partially purified Ah receptor preparations.

Isolation and receptor binding factor (RBF) activity of CP1–8. Non-histone chromatin protein fractions (CP1–8) were extracted from chromatin-hydroxylapatite using increasing concentrations of GdnHCl as described in Materials and Methods. From Fig. 2 it can be seen that the first few GdnHCl extractions removed a large percentage of the non-histone chromatin proteins. Subsequent extractions with GdnHCl removed decreasing amounts of chromatin proteins until only DNA-hydroxylapatite remained.

Reconstitution to dsDNA allows these highly hydrophobic chromosomal proteins to be soluble in low salt buffers and, therefore, allows for receptor binding assays. CP fractions 1, 2, 5, and 7 yielded the highest degree of RBF activity for [^{3}H]TCDD bound Ah receptor complexes when these CPs were reconstituted to rabbit dsDNA (Fig. 2). This binding represents total binding; however, non-specific binding to DNA alone was subtracted from all values. Since the Ah receptor preparation contained significant non-specific binding by [^{3}H]TCDD, we also measured AhRBF activity with [^{3}H]TCCD-receptor complexes prepared in the presence of 100-fold excess TCDF. As can be seen in Fig. 2, there was very little non-specific binding to NAPs 2–8.

Characteristics of [^{3}H]TCCD-receptor complex binding to putative receptor binding factors. Since CP 2, 5, and 7 exhibited a high degree of AhRBF activity we analyzed the binding kinetics of these fractions. The binding assay was performed using [^{3}H]TCDD-receptor complexes titrated over a 10-fold range of partially purified receptor preparations. Receptor binding factor activity of NAP 5 approached saturation (Fig. 3) for [^{3}H]TCDD-receptor complexes and a single class of high affinity sites was revealed by Scatchard analysis [23]. Similar results were obtained for NAP 2 and 7, i.e. RBF activity approached saturation (data not shown). Using the above binding assay conditions, estimates of the dissociation constants (K_d) were obtained from two experiments per CP fraction. Values were in the nanomolar range demonstrating high affinity binding of Ah receptor complexes to these reconstituted sites. Since these data were not corrected for the non-specific binding component present in the Ah receptor preparations, the K_d values are underestimated. The estimated number of binding sites per cell (B_{max}) ranged from approximately 1000 to 2000 for NAPs 2, 5, and 7 (Table 1). Binding of the labeled Ah receptor complexes to DNA alone was linear and non-saturable (not shown).

Specificity of Ah receptor binding to receptor

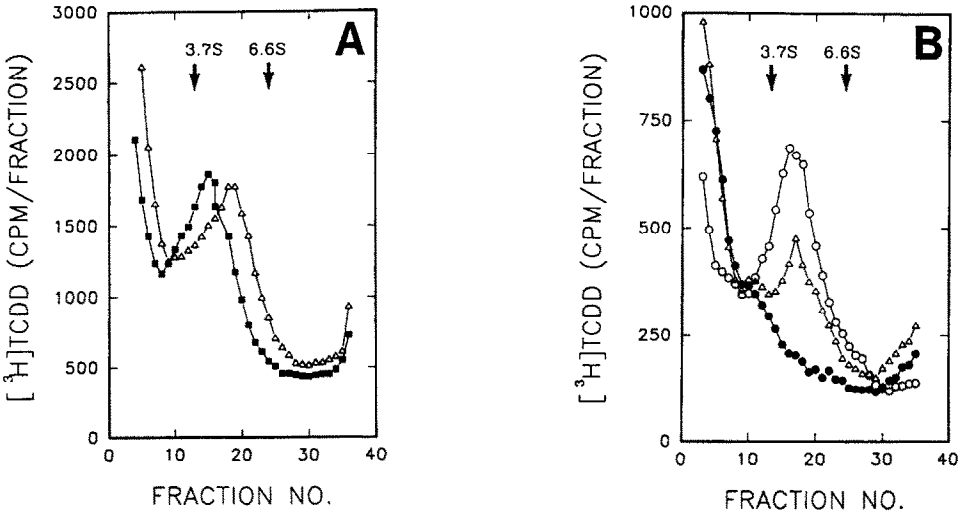

Fig. 1. Sucrose density gradient analysis of rabbit liver cytosolic [^{3}H]TCDD-receptor complexes. (A) Cytosol was prepared and incubated with 10 nM [^{3}H]TCDD as described in Materials and Methods. After DEAE step elution with 0.3 M KCl, 200 μL of the peak fraction was layered on a 5–20% sucrose density gradient prepared in 0.3 M KCl (■) or receptor preparation was adjusted to 0.15 M KCl and 200 μL layered on a 5–20% sucrose density gradient prepared in 0.15 M KCl (△). [^{14}C]γ-Globulin (6.6 S) and [^{14}C]ovalbumin (3.7 S) were used as sedimentation markers. Tubes were centrifuged at 225,000 g (SW 56 rotor) in a Beckman L5-50 ultracentrifuge for 16 hr. Gradient fractions were collected from the top, counted, and plotted as total bound cpm. (B) Sucrose density gradient analysis in 0.15 M KCl was performed as above except that cytosol was incubated with 10 nM [^{3}H]TCDD in the absence (△) or presence of 10 mM molybdate (○) or 200-fold excess TCDF (●).

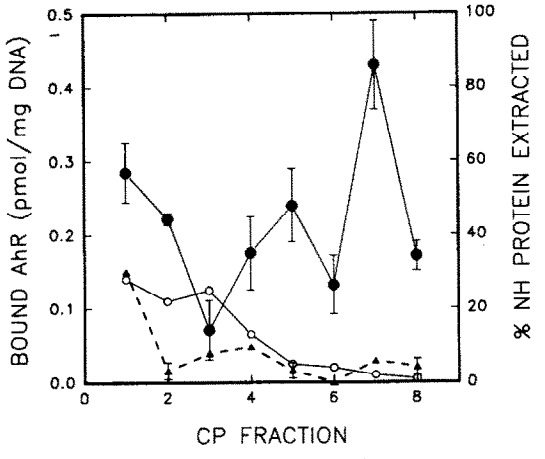

Fig. 2. Measurement of the binding of [^{3}H]TCDD-receptor complexes of CP fractions reconstituted to dsDNA. Samples of the extracted CPs were reconstituted to rabbit dsDNA by reverse gradient dialysis to generate nucleoacidic proteins (NAPs). Each NAP was probed with [^{3}H]TCDD-receptor complexes prepared in the absence (●) or presence (▲) of 100-fold excess TCDF to assay for receptor binding factor activity using the streptomycin filter assay. Binding to DNA was subtracted from these values. Also shown (○) is the protein profile of non-histone proteins extracted from rabbit liver chromatin by GdnHCl. One hundred per cent = 44 mg non-histone (NH) proteins. Data are expressed as means ± SEM for 3–4 determinations.

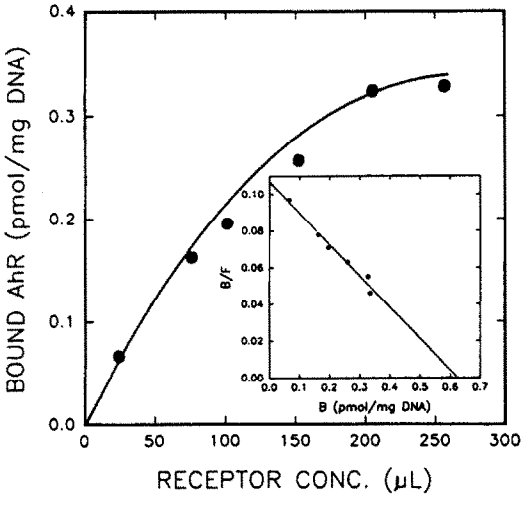

Fig. 3. Saturation kinetics for binding of [^{3}H]TCDD-receptor complexes to NAP obtained from CP5. Rabbit liver non-histone chromosomal proteins extracted with 5 M GdnHCl were reconstituted to dsDNA by reverse gradient dialysis. Increasing concentrations of [^{3}H]TCDD-receptor complexes were incubated with NAP equivalent to 70 μg DNA and binding was assayed. Data were also analyzed by Scatchard analysis [23].

Table 1. Ah receptor binding to chromatin receptor binding factors

Nucleoacidic protein	K_d (nM)	Bound Ah receptor (pmol/mg DNA)	Bound Ah receptor (sites/cell)
NAP 2*	3.1	0.70	~2500
NAP 5	0.5	0.62	~2200
NAP 7	1.0	0.33	~1200

* NAP = CP reconstituted to dsDNA.

binding factors in chromatin. To investigate the saturability and specificity of Ah receptor binding to AhRBFs, a competition experiment was performed. [^{3}H]TCDD-receptor complexes in the presence of 2-fold excess heat-denatured Ah receptor preparation, to keep protein concentration constant, were incubated with NAPs 2, 5, and 7 to measure total binding. Simultaneous experiments were also performed using [^{3}H]TCDD-receptor complexes plus a 2-fold excess of unlabeled TCDD-receptor complexes. Under these conditions the binding of [^{3}H]TCDD Ah receptor complexes to AhRBFs was decreased by 57% for NAP 7 (Fig. 4A). This reduction is near the theoretical maximum of 67% expected with a 2-fold excess addition of unlabeled receptor where the binding would be 100% specific. The binding in this experiment was shown to be 85% specific. Substantially lower binding was observed using DNA alone. Also, no competition was observed in the binding of [^{3}H]TCDD-receptor complexes to DNA alone (Fig. 4A); thus there was no specific binding to DNA. For NAPs 2 and 5 the specific binding was 61 and 59%, respectively (Fig. 4B).

Effect of molybdate on the binding of [^{3}H]TCDD-receptor complexes to RBFs. Ah receptor complexes were prepared in the presence and in the absence of 10 mM molybdate and then assayed for binding to receptor binding factors in CP fractions 2, 5, and 7 (Fig. 5). The binding obtained with [^{3}H]TCDD-receptor complexes prepared in the absence of molybdate was set equal to 100%. The binding of [^{3}H]TCDD-receptor complexes, prepared in the presence of 10 mM molybdate, to AhRBFs was reduced markedly (i.e. at least an 80% reduction) under this condition, suggesting non-transformed receptor.

Since the effect of molybdate on the Ah receptor appears to be variable depending on the species and procedures used to analyze the receptor [28, 29], we further examined the characteristics of the partially purified Ah receptor prepared in molybdate. Sucrose density gradients were performed using an aliquot of [^{3}H]TCDD-receptor complexes prepared in 10 mM molybdate and run on sucrose density gradients also prepared in 10 mM molybdate. In gradients containing 0.3 M KCl, the Ah receptor sedimented at 4 S (not shown), while in 0.15 M KCl the receptor was 6 S (Fig. 1B). These sedimentation values are similar to those obtained for estrogen receptor complexes prepared in 10 mM molybdate, partially purified by DEAE-Sephadex chromatography [30], and shown to be non-transformed. Others have also suggested that dissociation of heat-shock proteins from the native receptor is not sufficient to cause the complete transformation of steroid receptors [31]. In the present study molybdate appears to have altered the rabbit liver Ah receptor since the data presented in Fig. 5 clearly demonstrate that binding to AhRBFs was reduced dramatically.

DISCUSSION

The present experiments characterize the binding

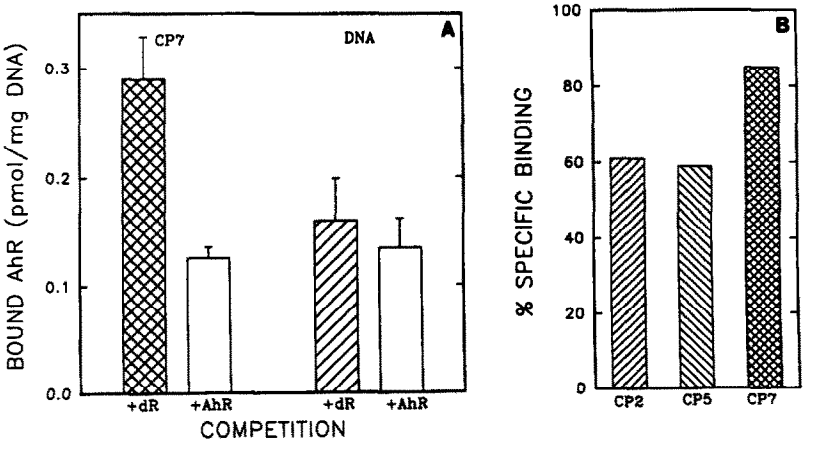

Fig. 4. (A) Competitive binding of Ah receptor to reconstituted NAP from CP7. Competition experiments were performed in which partially purified [^{3}H]TCDD-receptor complexes were used to determine binding to NAP from CP7 in the absence and in the presence of 2-fold excess unlabeled TCDD-receptor complexes. Protein content was kept constant with heat-denatured Ah receptor complexes (dR). The same experiment was performed, using dsDNA alone. Data are expressed as means ± SEM of 3–4 determinations. (B) Competition experiments performed as above except that data are expressed as per cent of specific binding for NAPs from CP2, CP5, and CP7.

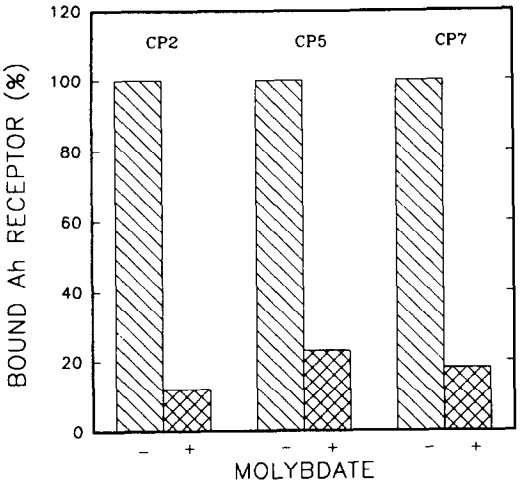

Fig. 5. Effect of molybdate on the binding of [^{3}H]TCDD-receptor to NAPs 2, 5, and 7. Ah receptor complexes were prepared in the presence (+) or in the absence (−) of 10 mM molybdate and assayed for binding to NAPs 2, 5, and 7. One hundred per cent bound Ah receptor = 0.1 pmol/mg DNA.

of [^{3}H]TCDD-receptor complexes to putative receptor binding factors in chromatin. Enhanced AhRBF activity was observed in three fractions of non-histone chromatin proteins, namely CP 2, 5, and 7, extracted from chromatin using increasing concentrations of GdnHCl. The binding of [^{3}H]-TCDD-receptor to these AhRBFs was shown to be saturable and of high affinity with estimated K_d values in the nanomolar range. The observed binding displayed specificity in competition experiments in which a 2-fold excess of unlabeled TCDD-receptor complexes reduced labeled Ah receptor binding. It is interesting that when Ah receptor was prepared in the presence of 10 mM molybdate, the binding of [^{3}H]TCDD-receptor to the AhRBFs was greatly reduced ($\geq$ 80%). This suggests that the binding to the NAPs requires a transformed Ah receptor complex. It has been shown that Ah receptor, like steroid hormone receptors, can be stabilized in a non-DNA binding form by sodium molybdate [4]. Analogously, it has been shown that steroid hormone receptor binding to chromatin or DNA requires transformed receptor complexes [32].

Specific chromatin proteins involved in the high affinity binding of steroid receptors within nuclei have also been reported by several other laboratories [33–38]. Previous studies in our laboratory [39, 40] demonstrated that rabbit uterine CP fractions extracted with 2, 5, and 7 M GdnHCl when reconstituted to DNA and probed with [^{3}H]estradiol-receptor complexes, exhibit estrogen RBF activity. Further characterization of estrogen RBFs in this laboratory have focused on CP5 because it exhibited RBF activity for both estrogen and antiestrogen bound estrogen receptors. Molecular seive chromatography resulted in a fraction (mol. wt approximately 12,000) with RBF activity for [^{3}H]estradiol-receptor complexes whereas another fraction (~30 kDa) demonstrated RBF activity for the receptor bound by the tamoxifen-like antiestrogen, H1285 [39]. Progesterone RBFs for avian progesterone receptor have also been isolated and studied [16]. Spelsberg and co-workers have purified one protein, termed RBF-1, to apparent homogeneity and identified others (RBF-2 and RBF-3) [41, 42].

Our laboratory [14] and others [19] have shown that the RBFs for rabbit estrogen receptors and avian progesterone receptors, respectively, exhibit target tissue specificity. That is, only in tissues responsive to hormone is RBF activity detectable. Additionally our laboratory has shown [15] in a study of antiestrogen-sensitive and antiestrogen-resistant MCF-7 cell lines that a certain antiestrogen-RBF present in the sensitive cell line is absent in the resistant cell line.

Dioxin is known to exhibit tissue specific effects [1]. These effects include lymphoid involution, especially of the thymus, changes in immunocompetence [reviewed in Ref. 43] and epithelial hyperplasia [1]. Given that dioxin has a multiplicity of effects [1], and also that dioxin has been shown to have antiestrogenic effects [44–47], we speculate that the AhRBFs discussed here may play a role in the multiplicity, tissue specificity, and antiestrogenic effects of TCDD. The antiestrogenic activity of TCDD and its congeners has been shown to correlate with the affinity of the various ligands for the Ah receptor [47]. It has been proposed that the antiestrogenic effects of TCDD are mediated through the Ah receptor [48]. This is of interest when considering our observations that chromatin protein fractions extracted with 2, 5, and 7 M GdnHCl exhibited a high degree of RBF activity for estrogen receptor binding to estrogen RBFs extracted from uterus and for Ah receptor binding to AhRBFs extracted from liver. It has been shown that Ah receptor is present in the uterus [46, 48]. Therefore, one possible mechanism of the antiestrogenicity of TCDD might be Ah receptor competing with estrogen receptor for estrogen RBFs in estrogen target tissues. This potential interaction would antagonize estrogenic responses in uterus such as wet weight increase or the increase in uterine peroxidase activity, both of which have been shown to be antagonized by an Ah receptor mediated mechanism [47].

A working model of our hypothesis involves a DNA looping phenomenon. In our model the heterodimeric Ah receptor initially binds an AhRBF in chromatin with high affinity and specificity. We envision the AhRBF as a "docking" protein for Ah receptor in chromatin. This binding then allows Ah receptor to be in proper alignment for a high affinity interaction with a dioxin-responsive enhancer [49, 50]. It is possible that the RBFs are associated with the nuclear matrix [51, 52] which allows for DNA organization and DNA loop attachment [53]. Thus, our model would provide a greater degree of specificity for Ah receptor interaction with its cognate response elements which would be necessary for low level TCDD exposure to elicit aberrant hormonal responses [54] and may account for some of the myriad effects of dioxin.

Recently, studies in our laboratory [55] showed that binding of the salt-transformed estrogen receptor by the antiestrogen H1285 induced a different conformation of the receptor than receptor bound by estradiol. These observations support the concept that antiestrogen binding could affect monomer–dimer equilibrium, thus rendering the antiestrogen-receptor complex incapable of inducing complete estrogenic responses. It has been shown [56] that the cytosolic Ah receptor bound [^{3}H]methylcholanthrene ([^{3}H]MC) and [^{3}H]TCDD with nearly equal affinity, but TCDD was far more potent than MC in the induction of AHH [57]. A recent study [58] sought to determine if the difference in AHH induction potency could be accounted for by differing abilities of TCDD and MC to transform Ah receptor. Using a gel retardation assay it was found that the difference in transformation potency, as measured by receptor binding to an oligonucleotide with a single DRE, was not sufficient to account for the large difference in AHH inducibility. In light of our observations, the large difference in potency could be accounted for if MC-receptor complexes had a reduced affinity for the AhRBF, which could result in an impaired AHH induction response. Further analysis of the properties of AhRBFs and their relation to other RBF families such as for the estrogen receptor or progesterone receptor should provide more insight into their role in mediating responses to TCDD.

Acknowledgements—This work was supported in part by NIH HD 13425. R.T.D. was supported in part by Training Grant NIH NS 07254.

REFERENCES

1. Poland A and Knutson JC, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* **22**: 517–554, 1982.
2. Safe S, Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu Rev Pharmacol Toxicol* **26**: 371–399, 1986.
3. Rucci G and Gasiewicz TA, *In vivo* kinetics and DNA-binding properties of the *Ah* receptor in the golden Syrian hamster. *Arch Biochem Biophys* **265**: 197–207, 1988.
4. Denis M, Wilhelmsson A, Cuthill S, Poellinger L and Gustafsson J-A, Structural differences between the glucocorticoid, dioxin and oxysterol receptors from rat liver cytosol. *Biochem Biophys Res Commun* **163**: 444–451, 1989.
5. Dougherty JJ, Separation and characterization of 7 and 9 S forms of rat liver aryl hydrocarbon receptor. *J Biol Chem* **264**: 8786–8790, 1989.
6. Cuthill S, Hapgood J, Denis M, Wilhelmsson A, Mason G, Nemeto T, Widmark E, Poellinger L and Gustafsson J, Ligand-dependent interaction of the dioxin receptor with target DNA. *J Steroid Biochem* **34**: 375–377, 1989.
7. Hapgood J, Cuthill S, Denis M, Poellinger L and Gustafsson J, Specific protein–DNA interactions at a xenobiotic-responsive element: Copurification of dioxin receptor and DNA-binding activity. *Proc Natl Acad Sci USA* **86**: 60–64, 1989.
8. Denison MS, Fisher JM and Whitlock JP Jr, Inducible receptor-dependent protein–DNA interactions at a dioxin-responsive element. *Proc Natl Acad Sci USA* **85**: 2528–2532, 1988.
9. Denison MS, Fisher JM and Whitlock JP Jr, The DNA recognition sequence for the dioxin-Ah receptor complex. *J Biol Chem* **263**: 17221–17224, 1988.
10. Whitlock JP Jr, Denison S, Fisher JM and Shen ES, Induction of hepatic cytochrome P450 gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol Biol Med* **6**: 169–178, 1989.
11. Hoffman EC, Reyes H, Chu F-F, Sander F, Conley LH, Brooks BA and Hankinson O, Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252**: 954–958, 1991.
12. Gasiewicz TA, Elferink CJ and Henry EC, Characterization of multiple forms of the Ah receptor: Recognition of a dioxin-responsive enhancer involves heteromer formation. *Biochemistry* **30**: 2909–2916, 1991.
13. Burbach KM, Poland A and Bradfield CA, Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci USA* **89**: 8185–8189, 1992.
14. Singh RK, Ruh MF and Ruh TS, Binding of [^{3}H]-estradiol- and [^{3}H]H1285-receptor complexes to rabbit uterine chromatin. *Biochim Biophys Acta* **800**: 33–40, 1984.
15. Singh RK, Ruh MF, Butler WB and Ruh TS, Characteristics of chromatin acceptor sites for estrogen- and antiestrogen-receptor complexes in sensitive and resistant MCF-7 cells. *Endocrinology* **118**: 1087–1095, 1986.
16. Spelsberg TC, Littlefield BA, Seelke R, Martin-Dani G, Toyoda H, Boyd-Leinen P, Thrall C and Kon OL, Role of specific chromosomal proteins and DNA sequences in the nuclear binding sites for steroid receptors. *Recent Prog Horm Res* **34**: 463–517, 1983.
17. Rories C and Spelsberg TC, Ovarian steroid action on gene expression: Mechanisms and models. *Annu Rev Physiol* **51**: 653–681, 1989.
18. Vos JG and Beems RB, Dermal toxicity studies of technical polychlorinated biphenyls and fractions thereof in rabbits. *Toxicol Appl Pharmacol* **19**: 617–633, 1971.
19. Spelsberg TC, Steggles AW and O'Malley BW, Progesterone-binding components of chick oviduct. *J Biol Chem* **247**: 1368–1374, 1972.
20. Spelsberg TC, Webster R, Pickler G, Thrall C and Wells D, Role of nuclear proteins as high affinity sites ("Acceptors") for progesterone in the avian oviduct. *J Steroid Biochem* **7**: 1091–1101, 1976.
21. Spelsberg TC, A rapid method for analysis of ligand binding to deoxyribonucleic acid and soluble nucleoproteins using streptomycin: Application to steroid receptor ligands. *Biochemistry* **22**: 13–21, 1983.
22. Burton K, A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* **62**: 315–323, 1956.
23. Scatchard G, The attraction of protein for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
24. Perdew GH, Chemical cross-linking of the cytosolic and nuclear forms of the *Ah* receptor in hepatoma cell line 1c1c7. *Biochem Biophys Res Commun* **182**: 55–62, 1992.
25. Wilhelmsson A, Cuthill S, Denis M, Wikstrom AC, Gustafsson JA and Poellinger L, The specific DNA binding activity of the dioxin receptor is modulated by the 90 kD heat shock protein. *EMBO J* **9**: 69–76, 1990.
26. Prokipcak RD and Okey AB, Physicochemical characterization of the nuclear form of *Ah* receptor from mouse hepatoma cells exposed in culture to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch Biochem Biophys* **267**: 811–828, 1988.
27. Mason ME and Okey AB, Cytosolic and nuclear binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the

Ah receptor in extra-hepatic tissues of rats and mice. *Eur J Biochem* **123**: 209–215, 1982.
28. Henry EC, Rucci G and Gasiewicz TA, Characterization of multiple forms of the Ah receptor: Comparison of species and tissues. *Biochemistry* **28**: 6430–6440, 1989.
29. Denison MS, Vella LM and Okey AB, Hepatic *Ah* receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Partial stabilization by molybdate. *J Biol Chem* **261**: 10189–10195, 1986.
30. Ruh MF and Ruh TS, Analysis of two forms of the molybdate stabilized estrogen receptor. *Endocrinology* **115**: 1341–1349, 1984.
31. Bagchi MK, Tsai M-J, O'Malley BW and Tsai SY, Analysis of the mechanism of steroid hormone receptor dependent gene activation in cell-free systems. *Endocr Rev* **13**: 525–535, 1992.
32. Nishigori H and Toft DO, Inhibition of progesterone receptor activation by sodium molybdate. *Biochemistry* **19**: 77–83, 1980.
33. Bagchi MK, Tsai SY, Tsai MJ and O'Malley BW, Purification and characterization of chicken ovalbumin gene upstream promoter transcription factor from homologous oviduct cells. *Mol Cell Biol* **7**: 4151–4158, 1987.
34. Edwards DP, Kuhnel B, Estes PA and Nordeen SK, Human progesterone receptor binding to mouse mammary tumor virus deoxyribonucleic acid: Dependence on hormone and nonreceptor nuclear factor(s). *Mol Endocrinol* **3**: 281–291, 1989.
35. Okamoto K, Isohashi F, Ueda K, Kokufu I and Sakamoto Y, Purification and characterization of an adenosine triphosphate-stimulated factor that enhances the nuclear binding of activated glucocorticoid-receptor complex from rat liver. *Endocrinology* **123**: 2752–2761, 1988.
36. Rennie PS, Bowden J-F, Bruchovsky N, Frenette P-S, Foekens JA and Cheng H, DNA and protein components of nuclear acceptor sites for androgen receptors in the rat prostate. *J Steroid Biochem* **27**: 513–520, 1987.
37. Ogle TF, Nuclear acceptor sites for progesterone receptor complexes in rat placenta. *Endocrinology* **121**: 28–35, 1987.
38. Cobb AD and Leavitt WW, Characterization of nuclear acceptor sites for mammalian progesterone receptor: Comparison with the chick oviduct system. *Gen Comp Endocrinol* **67**: 214–220, 1987.
39. Ruh MF and Ruh TS, Nuclear acceptor sites: Interaction with estrogen- virus antiestrogen-receptor complexes. In: *Steroid Receptors in Health and Disease* (Ed. Moudgil VK), pp. 233–250. Plenum Press, New York, 1988.
40. Ruh MF and Ruh TS, Specificity of chromatin acceptor sites for steroid hormone receptors. In: *Steroid Receptors and Disease* (Eds. Sheridan PJ, Blum K and Trachtenberg MC), pp. 23–48. Marcel Dekker, New York, 1988.
41. Schuchard M, Rejman JJ, McCormick DJ, Grosse B, Ruesink T and Spelsberg TC, Characterization of a purified chromatin acceptor protein (receptor binding factor 1) for the avian oviduct progesterone receptor. *Biochemistry* **30**: 4535–4542, 1991.
42. Landers JP and Spelsberg TC, New concepts in steroid hormone action: Transcription factors, proto-oncogenes, and the cascade model for steroid regulation of gene expression. *Crit Rev Euk Gene Exp* **2**: 19–63, 1992.
43. Holsapple MP, Morris DL, Wood SC and Snyder NK, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: Possible mechanisms. *Annu Rev Pharmacol Toxicol* **31**: 73–100, 1991.
44. Gallo MA, Hesse EJ, MacDonald GJ and Umbreit TH, Interactive effects of estradiol and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic cytochrome P-450 and mouse uterus. *Toxicol Lett* **32**: 123–132, 1986.
45. Romkes M and Safe S, Comparative activities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and progesterone as antiestrogens in the female rat uterus. *Toxicol Appl Pharmacol* **92**: 368–380, 1988.
46. Astroff B and Safe S, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin as an antiestrogen: Effect on rat uterine peroxidase activity. *Biochem Pharmacol* **39**: 485–488, 1990.
47. Safe S, Astroff B, Harris M, Zacharewski T, Dickerson R, Romkes M and Biegel L, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as antiestrogens: Characterization and mechanism of action. *Pharmacol Toxicol* **69**: 400–409, 1991.
48. Romkes M, Piskorska-Pliszczynska J and Safe S, Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on hepatic and uterine estrogen receptor levels in rats. *Toxicol Appl Pharmacol* **87**: 306–314, 1987.
49. Jones PBC, Galeazzi DR, Fisher JM and Whitlock JP Jr, Control of cytochrome P1-450 gene expression by dioxin. *Science* **227**: 1499–1502, 1985.
50. Sogawa K, Fujisawa-Sehara A, Yamane M and Fujii-Kuriyama Y, Location of regulatory elements responsible for drug induction in the rat cytochrome P450c gene. *Proc Natl Acad Sci USA* **83**: 8044–8048, 1986.
51. Schuchard M, Subramaniam M, Ruesink T and Spelsberg TC, Nuclear matrix localization and specific matrix DNA binding by receptor binding factor 1 of the avian oviduct progesterone receptor. *Biochemistry* **30**: 9516–9522, 1991.
52. Barrick ER, Steroid hormone receptor localization in the nuclear matrix: Interaction with acceptor sites. *J Steroid Biochem* **27**: 115–121, 1987.
53. Isaacs JT, Barrack ER, Isaacs WB and Coffey DS, The relationship of cellular structure and function: The matrix system. In: *The Prostatic Cell: Structure and Function* (Eds. Murphy GP, Sandberg A and Karr JP), pp. 1–24. Alan R. Liss, New York, 1981.
54. Mably TA, Moore RW and Peterson RE, *In utero* and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin 1. Effects on androgenic status. *Toxicol Appl Pharmacol* **114**: 97–107, 1992.
55. Ruh MF, Turner JW, Paulson CM and Ruh TS, Differences in the form of the salt transformed estrogen receptor when bound by estrogen versus antiestrogen. *J Steroid Biochem* **36**: 509–516, 1990.
56. Okey AB and Vella LM, Binding of 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to a common *Ah* receptor site in mouse and rat hepatic cytosols. *Eur J Biochem* **127**: 39–47, 1982.
57. Poland A and Glover E, Comparison of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a potent inducer of aryl hydrocarbon hydroxylase, with 3-methylcholanthrene. *Mol Pharmacol* **10**: 349–359, 1974.
58. Riddick DS, Harper PA and Okey AB, Comparative *in vitro* transformation of the aromatic hydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (MC). *FASEB J* **6**: A1201, 1992.