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Characterization of Multiple Forms of the Ah Receptor: Comparison of Species and Tissues†

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ABSTRACT: Biochemical and toxic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) appear to be mediated via the Ah receptor, a gene-regulatory protein that, like steroid hormone receptors, undergoes a ligand-dependent acquisition of affinity for nuclei and DNA. Since responses to TCDD are highly species- and tissue-specific, we compared DNA-binding properties of Ah receptor from several tissues of rat, C57BL/6 mouse, hamster, and guinea pig, using DNA–Sepharose chromatography. Hepatic cytosol from all species contained TCDD·receptor complexes that eluted at ~0.15 (peak 1) and ~0.33 M NaCl (peak 2). The relative proportions of these forms as well as of TCDD·receptor that did not bind to DNA (i.e., was present in flowthrough fractions) varied among species. In each case, the yield of the higher affinity form (peak 2) increased with time or temperature of incubation. Cytosol from lung, thymus, kidney, and testis contained the same two forms; peak 2 was the major DNA-binding form only in thymus. In KCl extracts of hepatic nuclei from animals treated with [^{3}H]TCDD, only the higher affinity form (peak 2) was found. Peak 1 isolated from cytosol by DNA–Sepharose and incubated with hepatic cytosol from D2 mouse (which contains no detectable receptor) transformed into peak 2, suggesting that these two forms are different conformations of the same protein. Sucrose density gradient and gel filtration analyses of peaks 1 and 2 isolated from DNA–Sepharose indicated that (i) the untransformed form (peak 1) was smaller than the unoccupied and the transformed forms, (ii) 0.4 M KCl in the density gradients had little effect on these isolated forms, and (iii) nuclear receptor sedimented like peak 2. On the basis of these results, we hypothesize that the Ah receptor exists in several forms: When occupied, it has no affinity for DNA. Ligand binding initially yields a smaller form with low DNA affinity (i.e., peak 1), as well as, in some cases, a form with no DNA affinity (flowthrough fractions); further incubation in the presence of cytosolic factor(s) induces a change conferring higher DNA affinity and faster sedimentation (i.e., peak 2). The latter form is likely the transcriptionally active form in vivo. Species and tissue differences in this scheme are quantitative rather than qualitative.

The Ah (*a*ryl *h*ydrocarbon) receptor is a soluble intracellular protein that binds numerous halogenated and nonhalogenated aromatic hydrocarbons and thereby appears to mediate many of the biochemical and toxic responses to these xenobiotics, of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[1] is the most potent (Poland & Knutson, 1982; Whitlock, 1987). Both physicochemically and functionally, the Ah receptor is analogous to steroid hormone receptors, although no endogenous ligand for the Ah receptor has yet been identified. The Ah and glucocorticoid receptors are strikingly similar in their molecular size, surface charge, and sedimentation coefficient (Wilhelmson et al., 1986; Cuthill et al., 1987). Binding of their respective ligands confers on both Ah and steroid receptors greater thermostability, greater resistance to disruption by reagents such as salt and SH modifiers, and higher affinity for cell nuclei and for DNA in vitro (Okey et al., 1979; Carlstedt-Duke et al., 1981; Schmidt & Litwack, 1982; Hannah et al., 1986; Kester & Gasiewicz, 1987; Henry et al., 1988). These ligand-induced changes are presumed to result from a conformational change(s) in the receptor protein. Most importantly, the Ah receptor, like the steroid receptors, is a gene regulatory protein: the ligand·receptor complexes have high affinity for nuclear material and modify expression of a receptor- and tissue-specific battery of genes (Whitlock, 1987).

In the case of the Ah receptor, the best-studied effect at the genetic level is the activation of the gene(s) encoding specific cytochrome P-450 isozymes (e.g., the gene P450IA1 encoding cytochrome P_1-450 in mouse, P-450c in rat) [reviewed by Eisen et al. (1983) and Jones et al. (1985)]. Tukey et al. (1982) demonstrated a direct correlation between the presence of the TCDD·receptor complex in the nucleus and synthesis of P_1-450 mRNA. Upstream from the P_1-450 gene, three dioxin-responsive elements that are transcriptional enhancers have been identified, and they are functionally dependent on the TCDD·receptor complex (Durrin & Whitlock, 1987; Denison et al., 1988). Although the induction of these P-450s and associated mixed-function oxidases by TCDD is well docu-

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[1] Abbreviations: BSA, bovine serum albumin; B6, C57BL/6J; D2, DBA/2J; HAP, hydroxylapatite; HEDG, 25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol (pH 7.6); hsp90, 90-kDa heat shock protein; PMSF, phenylmethanesulfonyl fluoride; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCDD ± TCDF, [^{3}H]TCDD ± 100-fold excess of TCDF.

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mented (Hook et al., 1975; Goldstein & Hardwick, 1984), it is unknown whether or not they are directly involved in TCDD's toxicity [e.g., Gasiewicz et al. (1986)]. However, the interactions of the TCDD·receptor complex with other genes that may mediate toxicity are presumably analogous.

The biochemical and toxicologic importance of receptor binding of TCDD and the subsequent nuclear association of the complex have been demonstrated by using receptor-defective mouse strains and cells [e.g., Poland et al. (1976), Israel and Whitlock (1983), and Karenlampi et al. (1988)]. It is not known, however, whether there are species differences in TCDD·receptor complex formation and nuclear association that could partially account for the marked species variability in biochemical responses to TCDD. Parameters of ligand binding such as K_d and b_{max} are quite similar among rats, mice, hamsters, guinea pigs, and monkeys despite the wide range of LD50s among these species (Gasiewicz & Rucci, 1984; Denison & Wilkinson, 1985). Additionally, physical characteristics of the receptor are generally similar among species, although some variation in molecular size has been detected [e.g., Denison et al. (1986a) and Poland and Glover (1987)]. Our previous studies (Gasiewicz & Bauman, 1987) indicated that in rat hepatic cytosol at least three forms of the Ah receptor can be distinguished by their net charge and affinity for DNA–Sepharose. Furthermore, only the form that has the lower net negative charge is detected in nuclear extracts from livers of [^{3}H]TCDD-treated rats. Since the conversion to a DNA-binding state presumably determines the amount of receptor available with the potential to modulate gene expression, it was of interest (1) to determine whether species- and tissue-specific differences are detectable in these forms of the receptor in both the cytosol and the nucleus and (2) to further characterize the process of transformation in vitro to a DNA-binding state and the relationships among these forms of the receptor.

Experimental Procedures

Chemicals. [^{3}H]TCDD (KOR Isotopes Inc.; approximately 35 Ci/mmol) was dissolved in *p*-dioxane and purified by high-pressure liquid chromatography by the method of Gasiewicz and Neal (1979). TCDF was obtained from the National Institute of Environmental Health Sciences. Calf thymus DNA and cyanogen bromide activated Sepharose 4B were purchased from Sigma, and Superose 6 (prep grade) was purchased from Pharmacia LKB. Other chemicals were from standard sources and of highest available grade.

Buffers. HEDG buffer consisted of 25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol, pH adjusted to 7.6 at room temperature. For some procedures, this buffer was modified as noted in figure legends.

Animals, Tissue Preparations. Male Sprague-Dawley rats (200–250 g), C57BL/6J and DBA/2J mice (20–25 g), Syrian golden hamsters (110–150 g), and Hartley guinea pigs (300–350 g) were used. Mice were purchased from Jackson Labs (Bar Harbor, ME); other species were from Charles River (Wilmington, MA). Animals were housed under laboratory conditions for at least 3 days prior to use and were fed commercial chow and water ad libitum. In experiments in which nuclear receptor was to be analyzed, animals were injected ip with [^{3}H]TCDD (20 μg/kg) or with 200 μg of TCDD/kg followed 4 h later by 20 μg of [^{3}H]TCDD/kg; these animals were killed 16 h later. Cytosol and nuclear extracts were prepared as described by Gasiewicz and Bauman (1987). [^{3}H]TCDD was added to cytosol in dioxane (5 μL/mL of cytosol) at a final concentration of 2 or 3 nM with and without a 100-fold excess of unlabeled TCDF (hereafter referred to as TCDD ± TCDF). Hepatic cytosols were adjusted to 15–20 mg of protein/mL (5 mg/mL for B6 mouse); cytosols from other tissues were 12–20 mg/mL (except thymus, 6–7 mg/mL). For removal of free and loosely bound ligand, all samples were treated with dextran/charcoal (0.05 mg/0.5 mg per milliliter of cytosol) for 10 min at 0–4 °C and filtered through Millex HV filters prior to chromatography. Further incubation conditions are specified in figure legends. Specific binding of [^{3}H]TCDD was also determined in each cytosol or nuclear extract by using hydroxylapatite (HAP) adsorption (Gasiewicz & Neal, 1982) for comparison with chromatographic analyses.

DNA–Sepharose Chromatography. Preparation of DNA–Sepharose, column packing, chromatographic conditions, elution with a NaCl gradient, and analysis of the eluted fractions were as described by Gasiewicz and Bauman (1987). Volumes of hepatic cytosol equivalent to 30–40 mg of protein were loaded onto columns. Nuclear extracts were diluted to approximately 0.05 M KCl, and 12–18 mg of protein was loaded onto each column. In all experiments, cytosols incubated with and without excess TCDF were chromatographed simultaneously on matched columns and yielded profiles of nonspecific and total binding, respectively. Only specific binding (calculated by subtraction of nonspecific from total binding in each fraction) is shown in the figures.

Sucrose Density Gradient Centrifugation. Dextran/charcoal-treated cytosols or pooled fractions representing specific binding peaks from DNA–Sepharose columns were layered onto 10–30% sucrose gradients prepared in HEDG or in HEDG plus 0.4 M KCl in Beckman Quick-Seal polyallomer tubes. [*methyl*-^{14}C]Methylated ovalbumin (3.6 S) and catalase (11.3 S) were added to each tube as sedimentation markers. Paired tubes (representing total and nonspecific binding) were centrifuged in a vertical tube rotor (Beckman, VTi80) at 304000g_{av} for 110 min at 4 °C, and 0.2-mL fractions were collected with an ISCO Model 640 density gradient fractionator. The position of catalase was determined enzymatically. For sedimentation analysis of unoccupied receptor, 200 μL of untreated cytosol (~20 mg of protein/mL) was centrifuged on gradients with and without KCl and fractionated (at 4 °C) as above; the appropriate fractions were sampled for the markers, and then 0.2 mL of HEDG buffer and 4 μL of TCDD ± TCDF (final concentration 0.5 nM [^{3}H]TCDD) were added to each 0.2-mL fraction. After overnight incubation on ice, specific binding was measured by the HAP assay. Control experiments indicated that the resulting concentrations of sucrose do not interfere with the HAP assay.

High-Resolution Gel Filtration. Superose 6 was packed into a HR 10/30 column (Pharmacia LKB) and eluted with 0.4 M NaCl in HED buffer (HEDG without glycerol) at 0.5 mL/min by use of the Pharmacia LKB FPLC system. The column was calibrated by using the following protein standards (R_S in nanometers): cytochrome *c* (1.79); albumin (3.59); apoferritin (6.15); thyroglobulin (8.5). A volume of 200 μL of sample (or standard) was injected, and 1-min fractions were collected.

Safety Precautions. Because of the high toxicity of TCDD and TCDF, all contaminated materials are collected separately for disposal by the Hazardous Waste Unit. Any glassware that is not disposable that is used for TCDD-contaminated samples is carefully washed (all waste water is charcoal filtered) and kept separate from other glassware. TCDD-treated animals are housed in a Hazardous Substance Facility.

Results

DNA–Sepharose Profiles of Hepatic Cytosols. Three peaks of specific binding of TCDD were observed in hepatic cytosols

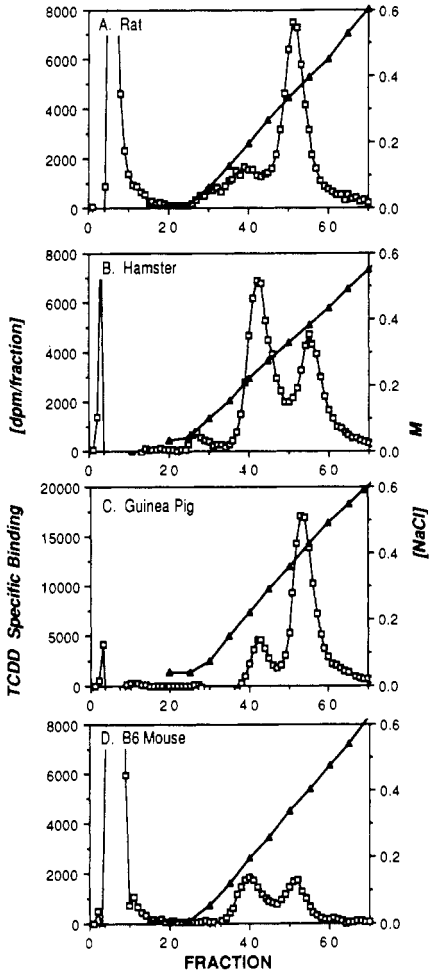

FIGURE 1: DNA–Sepharose profiles of hepatic cytosols after a 2-h incubation at 20 °C. Cytosols were prepared from perfused livers of rat (A), hamster (B), guinea pig (C), or B6 mouse (D) and were incubated with 2 or 3 nM TCDD ± TCDF. Unbound ligand was removed with dextran/charcoal, and cytosols were chromatographed on DNA–Sepharose columns eluted with HEDG and a NaCl gradient (▲). The specific binding shown was calculated by subtracting the dpm eluting in each fraction in the presence of a 100-fold excess of TCDF (nonspecific binding) from the dpm in the corresponding fraction from the parallel column in the absence of TCDF (total binding).

from guinea pig, B6 mouse, and hamster following a 2-h incubation at 20 °C (Figure 1) as previously described in rat liver by Gasiewicz and Bauman (1987). A variable amount of specifically and nonspecifically bound [^{3}H]TCDD eluted in the flowthrough fractions (i.e., did not bind to DNA) as discussed below; that which was retained on the DNA–Sepharose eluted at NaCl concentrations of 0.20–0.25 (peak 1)[2] and 0.33–0.39 M (peak 2)[2] in each species. The major variation among species was in the distribution of specific binding between peaks 1 and 2, as evident in Figure 1. No specific binding was detected on DNA–Sepharose (or by HAP) in cytosol from D2 mouse liver that was incubated under the same conditions (not shown).

We also examined profiles obtained when cytosols were incubated under different conditions (data not shown). When rat hepatic cytosol was incubated at 0 °C for 20 h, the profile was identical with that shown in Figure 1A. After only 2 h at 0 °C, more specific binding eluted in the flowthrough, and of that which bound to DNA–Sepharose, at least half was present as peak 1. Hamster receptor appeared to be more resistant to transformation[3] to peak 2 at 20 °C (cf. Figure 1B); longer incubation (4 h) at 20 °C yielded approximately equal amounts of specific binding in peaks 1 and 2, though this change was due mainly to a loss of peak 1 rather than an increase in peak 2. During incubation of hamster cytosol at 0 °C for 2, 20, or 46 h, there was some conversion from peak 1 to peak 2 with time, although peak 1 was generally predominant. In contrast, guinea pig receptor underwent rapid transformation to peak 2 at 0 °C; during 2, 20, or 46 h of incubation, peak 1 decreased progressively with a concomitant increase in peak 2. Similar to the rat receptor, incubation of guinea pig cytosol for 20 h at 0 °C resulted in a profile equivalent to that in Figure 1C after 2 h at 20 °C. In B6 mouse cytosol, both peaks 1 and 2 were notably small compared with other species (Figure 1D); also, 50–80% of the total eluted specific binding was detected in the flowthrough fractions compared to 0% in guinea pig and 10–30% in rat and hamster (Table I). These peak sizes and distribution were unchanged by incubation overnight at 0 °C. Because of the higher K_d of the B6 mouse receptor compared to other species (Gasiewicz & Rucci, 1984), a 3-fold higher concentration of TCDD per milligram of protein was tested, but this did not alter the degree of retention on DNA–Sepharose.

Table I: Species Differences in [^{3}H]TCDD Specific Binding Eluting in Flowthrough Fractions from DNA–Sepharose[a]

species	specific binding in flowthrough fractions as percent of total eluted specific binding ± SD (*n*)
rat	29 ± 6 (7)
hamster	13 ± 7 (5)
guinea pig	0 ± 0 (4)
B6 mouse	79 ± 2 (3)

[a] Hepatic cytosols from each species were incubated with TCDD ± TCDF for 2 h at 20 °C, treated with dextran/charcoal, and chromatographed on DNA–Sepharose as described under Experimental Procedures. Total eluted specific binding was calculated as the sum of specific binding in all fractions from the parallel columns (difference between dpm/fraction in cytosols incubated with TCDD in the absence and in the presence of TCDF). The amount of specific binding that appeared in fractions 1–16 (flowthrough) is expressed as a percentage of the total. In all cases, approximately the same amounts of total protein and total [^{3}H]TCDD were applied to the columns.

Unoccupied receptor from each species, measured as described by Gasiewicz and Bauman (1987), eluted from the columns only in the flowthrough fractions (data not shown), indicating that only ligand-bound receptor was able to bind to DNA–Sepharose. Furthermore, when unoccupied receptor was heat-treated (1 h at 20 °C) prior to chromatography, it still eluted only in flowthrough fractions, demonstrating that heating does not confer affinity for DNA in the absence of ligand.

DNA–Sepharose Profiles of Cytosol from Selected Tissues. The above results demonstrated that in each species the hepatic receptor existed in several forms, i.e., unoccupied and/or ligand-bound but non-DNA-binding and two occupied forms differing in affinity for DNA. The relative quantities of these

[2] Preparations of DNA–Sepharose differed slightly in their affinity for the Ah receptor·ligand complex (i.e., NaCl concentrations at which peaks 1 and 2 eluted varied among batches). For each batch, elution concentrations were consistent among experiments; however, since the experiments shown were performed on several batches of the gel, these values vary somewhat among figures. In particular, except for Figure 1B–D, most results were obtained on columns from which peaks 1 and 2 typically eluted at 0.15–0.18 and 0.32–0.36 M NaCl, respectively.

[3] We use the term "transformation" to describe the changes in the receptor·ligand complex that confer higher affinity for DNA. Thus, peak 1, which has lower DNA affinity, is referred to as "untransformed" receptor and peak 2 as "transformed" receptor.

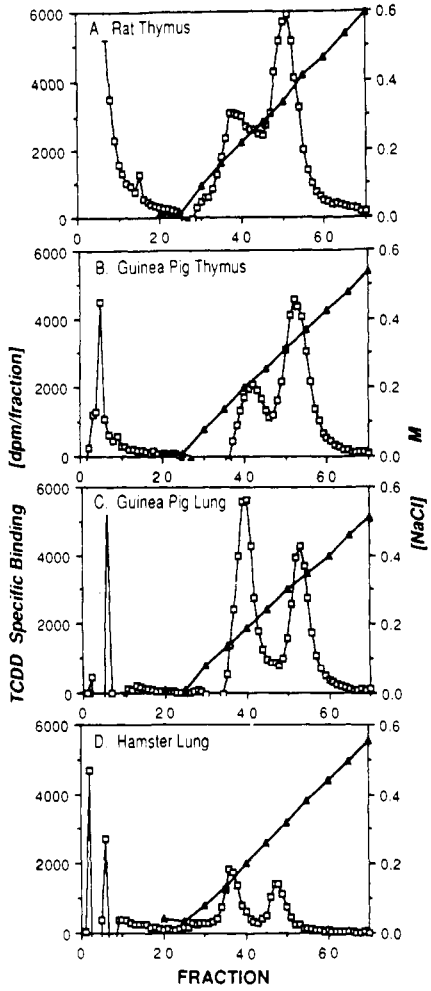

FIGURE 2: DNA–Sepharose profiles of cytosols from thymus and lung after a 2-h incubation at 20 °C. Cytosols were prepared from rat thymus (A), guinea pig thymus (B), guinea pig lung (C), or hamster lung (D) and treated as indicated for Figure 1.

forms varied among species and incubation conditions. We therefore investigated whether similarities and differences can be detected in other tissues from these species. Nonhepatic tissues in which substantial levels of specific binding were detected by using the HAP assay (Gasiewicz & Rucci, 1984) were chosen from each species for DNA–Sepharose analysis. In all cases, two peaks eluted at NaCl concentrations equal to those of peaks 1 and 2 in hepatic cytosol. The amount of specific binding in flowthrough fractions was variable among tissues and species. In thymus from guinea pig and rat, peak 2 was the major DNA-binding form (Figure 2A,B). In lung cytosol from guinea pig and hamster (Figure 2C,D) as well as kidney and testicular tissue (not shown), peak 1 was of equal or larger size than peak 2.

DNA–Sepharose Profiles of Hepatic Nuclear Extract from [^{3}H]TCDD-Treated Animals. Since presumably only ligand·receptor complex that is in the nucleus can directly affect gene expression, it was of interest to determine which of the DNA-binding forms of TCDD·receptor is found in the nucleus after in vivo exposure to TCDD. Specific binding in KCl extracts of hepatic nuclei from [^{3}H]TCDD-treated guinea pig, hamster, rat, B6 mouse, and also D2 mouse eluted from DNA–Sepharose as a single sharp peak at a NaCl concentration characteristic of peak 2; no significant amounts of specific binding were found in the flowthrough fractions. The profiles for all species were qualitatively similar to that shown for guinea pig in Figure 3. This detection of a single peak was not an artifact of the high-salt extraction procedure, since

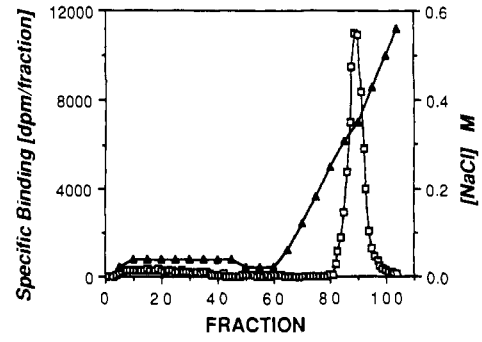

FIGURE 3: DNA–Sepharose profile of nuclear extract of guinea pig liver. One guinea pig was dosed ip with 20 μg of [^{3}H]TCDD and a second one with 200 μg of TCDD 4 h prior to the dose of 20 μg of [^{3}H]TCDD. After 16 h, nuclei were isolated from the livers of both animals, and TCDD·receptor complexes were extracted with 0.5 M KCl (see Experimental Procedures). Nuclear extracts were diluted with HEDG buffer to approximately 0.05 M KCl prior to chromatography on parallel DNA–Sepharose columns as described in Figure 1. The first 40 fractions shown are those collected during the loading of the large volume of diluted nuclear extract. In vivo labeling and preparation of nuclear extracts from each species were the same, except for DBA mice, in which 40 μg of [^{3}H]TCDD was administered.

0.5 M KCl treatment of rat cytosol after 1 h of incubation with TCDD at 0 °C (at which time there is very little of peak 2) and of hamster cytosol (in which peaks 1 and 2 are of approximately equal magnitude) had no effect on DNA–Sepharose profiles (not shown).

Specific Binding in Flowthrough Fractions. As mentioned above, one of the parameters that varied among species was the proportion of specifically bound TCDD in cytosol that did not bind to DNA–Sepharose, a summarized in Table I. The general species differences were consistent, although they depended somewhat on cytosolic incubation conditions and on the batch of DNA–Sepharose used[2] (not shown).

Although our protocols achieved a greater degree of TCDD·receptor complex binding to DNA than reported previously with DNA–cellulose (Carlstedt-Duke et al., 1981; Hannah et al., 1986; Wilhelmsson et al., 1986; Cuthill & Poellinger, 1988), the reason for high amounts in the flowthrough in some cases is not clear. One possibility is that receptors aggregate, perhaps blocking DNA-binding domains, and/or are associated with charged molecules such as RNA that may compete for DNA-binding sites. Therefore, we examined DNA–Sepharose profiles after treatment of the TCDD-bound, transformed receptor with (1) 0.3 M KCl, which would presumably dissociate most ionic interactions in protein aggregates, or (2) RNase (20 units/mL). These agents were added after a 2-h incubation of rat cytosol at 20 °C with TCDD ± TCDF for a further incubation at 0 °C (30 min, KCl) or 20 °C (2 h, RNase). KCl-treated cytosol was diluted to 0.03 M just prior to chromatography (control was diluted to the same volume). Treatment with 0.3 M KCl did not enhance retention on DNA–Sepharose compared with diluted control cytosol to which 0.03 M KCl was added for loading (data not shown). However, addition of KCl (0.05 M) just prior to chromatography of normal volumes of cytosol (2–3 mL) decreased the proportion of specific binding eluting in the flowthrough from 25 to 12% in two experiments, relative to control cytosol without KCl, and concomitantly increased the proportion retained and eluted by the NaCl gradient as peak 2. In B6 mouse hepatic cytosol, in which retention of specific binding on DNA–Sepharose is very low (Table I), the 0.3 M KCl protocol described above caused a modest increase in retention (from 25 to 36% of total eluted specific binding), but this still did not approach the control levels observed in

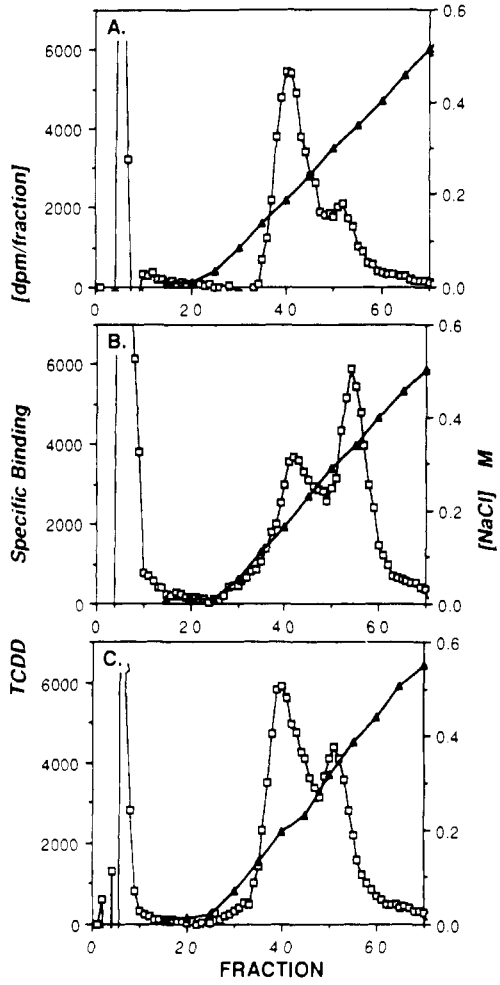

FIGURE 4: Reconstitution of receptor transformation in charcoal-treated rat hepatic cytosol by hepatic cytosol from D2 mouse. Rat hepatic cytosol (~24 mg of protein/mL) was incubated with activated charcoal (4% w/v, pelleted from slurry in distilled water) for 15 min at 0 °C. Charcoal was removed by centrifugation followed by filtration through Millex HV filters. The resulting cytosol (~18 mg of protein/mL), was supplemented with 5 mM dithiothreitol and incubated with 3 nM TCDD ± TCDF at 0 °C for 18 h. In (A), DNA–Sepharose chromatography was performed as described in Figure 1. In (B), 3-mL portions of the incubated, TCDD-labeled cytosol were mixed with 1 mL of cytosol from D2 mouse liver (20 mg of protein/mL before mixing); to other 3-mL portions of the same cytosol was added 1 mL of HEDG buffer (C). These mixtures were incubated for 2 h at 20 °C and then chromatographed as described in Figure 1.

the other species. Thus, receptor aggregation that is salt inhibitable appears to only partially account for the presence of non-DNA-binding receptor. RNase treatment of the TCDD-labeled rat hepatic cytosol also decreased the flow-through specific binding by approximately 50%, with a corresponding increase in retention of transformed receptor on DNA (data not shown). A similar effect of RNase on the glucocorticoid receptor has been reported by Schmidt et al. (1986). These results suggest that the presence of specifically bound TCDD in the flowthrough may partially reflect receptor association with proteins and other molecules including RNA, although since we are using crude cytosol, it is not discernible whether this is a specific association as reported for the glucocorticoid receptor–RNA interaction (Ali & Vedekis, 1987). It is also possible that RNA in crude cytosol competes with TCDD-receptor for binding sites on the DNA–Sepharose.

Evidence That the DNA-Binding Forms Are Different Conformations of the Same Protein. In other studies, we had observed that pretreatment of rat hepatic cytosol with activated charcoal prior to incubation with TCDD (18 h at 0 °C) resulted in specific binding eluting from DNA–Sepharose almost exclusively as peak 1 (Figure 4A), although there was no effect on specific binding as measured by HAP (Henry and Gasiewicz, unpublished results). However, transformation in this charcoal-treated cytosol could be reconstituted after labeling with TCDD by adding hepatic cytosol from D2 mouse for a further 2-h incubation at 20 °C (Figure 4B). Note that incubation of charcoal-treated cytosol alone for 2 h at 20 °C enabled only a small degree of transformation to peak 2 to occur (Figure 4C). Since D2 cytosol itself contains no detectable receptor under these conditions, peak 2 in Figure 4B represents material that eluted as peak 1 in Figure 4C and underwent transformation in the presence of D2 cytosol, suggesting that peak 1 is indeed an intermediate conformation of the same protein that elutes as peak 2. Addition of D2 cytosol had no effect on specific binding assayed by HAP or on elution profiles of control rat cytosol (not charcoal-treated). The same shift of peak 1 to peak 2 in charcoal-treated rat cytosol could be achieved by mixing with rat cytosol in which receptor had been saturated by incubation (18 h, 0 °C) with TCDF (data not shown).

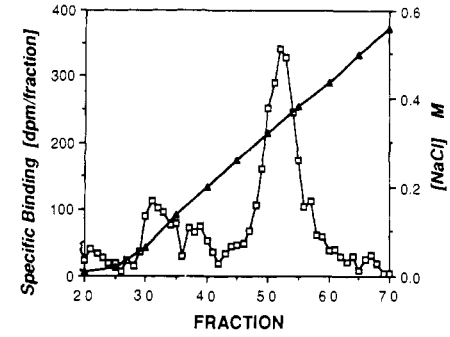

FIGURE 5: Transformation by D2 mouse hepatic cytosol of peak 1 isolated from DNA–Sepharose. Guinea pig hepatic cytosol (17 mg of protein/mL) was incubated with 3 nM TCDD ± TCDF for 2 h at 0 °C and chromatographed on DNA–Sepharose as described for Figure 1. Four fractions of maximum radioactivity representing peak 1 were pooled, diluted with HEDG buffer to reduce [NaCl]; D2 hepatic cytosol was added (2.3 mL at 17 mg of protein/mL prior to mixing) for a further incubation for 2 h at 20 °C. This mixture was then chromatographed on DNA–Sepharose, as described for Figure 1. No net specific binding appeared in the flowthrough fractions (1–18). Recovery of specific binding was ~28% of the specific binding obtained as peak 1 from the first chromatography; much of this loss likely reflects adsorption onto the glass collection and incubation vessels.

Inhibition of transformation by pretreatment with activated charcoal and reconstitution with D2 cytosol were also observed in hepatic cytosol of hamster, guinea pig, and B6 mouse (not shown). Thus, in each species, charcoal removed some cytosolic factor(s) that normally mediate the process of transformation, and the factor(s) in D2 cytosol was (were) effective in transforming the receptor in all species tested. Preliminary observations indicate that this factor is a heat-labile protein (unpublished results).

More direct evidence of the conversion of peak 1 to peak 2 is presented in Figure 5. In this protocol, guinea pig hepatic cytosol was incubated with TCDD ± TCDF (2 h, 0 °C) prior to DNA–Sepharose chromatography. Four fractions representing peak 1 were pooled and diluted from 0.15 to 0.08 M NaCl by adding HEDG buffer and D2 mouse cytosol for further incubation at 20 °C for 2 h. Rechromatography of this mixture on DNA–Sepharose yielded specific binding eluting principally as peak 2, at 0.33 M NaCl (Figure 5). Addition of charcoal-inactivated D2 cytosol to pooled peak 1 fractions did not facilitate this transformation to peak 2 (not shown). Thus, the transformed receptor can be obtained from the isolated peak 1, although this conversion requires additional

cytosolic factor(s). The finding that peak 1 is not present upon DNA–Sepharose analysis of nuclear receptor (Figure 3) or of the transformed receptor obtained from DEAE-Sepharose (Gasiewicz & Bauman, 1987) or DNA–Sepharose (not shown) indicates that transformation to peak 2 is not an easily reversible process.

Evidence That Peak 2 Is Not a Proteolytic Degradation Product of Peak 1. Poland et al. (1986) reported that a photoaffinity ligand for the Ah receptor labels both a 70- and a 95-kDa peptide in mouse liver cytosol. Subsequent studies in Hepa 1 cells (Poland & Glover, 1988) indicated that a calcium-activated proteinase in the cytosol hydrolyzes the 95-kDa peptide to yield the 70-kDa fragment and that this process is inhibited by the proteinase inhibitor leupeptin. Additionally, calpastatin, the endogenous inhibitor of calcium-dependent proteinases, has been found to stabilize the thymic glucocorticoid receptor (Bodwell et al., 1985). Estrogen receptor transformation (as defined by sedimentation properties) has also been attributed to a proteolytic activity (Notides et al., 1973; Puca et al., 1977, 1986). Therefore, to test the hypothesis that the observed "transformation" of Ah receptor from peak 1 to peak 2 is an artifact of proteolytic activity, we determined the effects of $CaCl_2$ (as a stimulator of proteinases) and of two proteinase inhibitors on the DNA–Sepharose profile of the Ah receptor.

Neither 5 mM leupeptin nor 1 mM PMSF had any significant effect when added during a 2-h, 20 °C incubation of rat hepatic cytosol (not shown). Similarly, leupeptin did not modify the ability of D2 cytosol to effect transformation of charcoal-treated rat receptor. Since hamster receptor remains as primarily peak 1 even after 2 h at 20 °C (cf. Figure 1), we added 3 mM $CaCl_2$ during such an incubation to determine whether calcium-activated proteinases might enhance the transformation to peak 2. This concentration of calcium was apparently detrimental to the hamster receptor as there was an approximately 40% loss of specific binding as measured by HAP, and rather than enhancing transformation to peak 2, calcium treatment eliminated peak 2 and broadened peak 1 (not shown). Likewise, calcium did not increase the extent of DBA-induced transformation of charcoal-treated rat receptor, but instead essentially eliminated peak 2 and diminished peak 1. Thus, these results support the conclusion that the transformation from peak 1 to peak 2 is not simply a proteolytic breakdown analogous to that observed by Poland and Glover (1988) but likely has biological significance; it is possible, however, that other specific proteinases which are not calcium-activated or not affected by these inhibitors may act on the Ah receptor.

After these experiments were completed, we found that peak 1 is the smaller of the two DNA-binding forms, as defined by sedimentation coefficient and Stokes radius (Tables II and III), substantiating the conclusion that transformation is not simply a proteolytic process. The possibility that peak 1 represents a proteolytic product of peak 2 is unlikely, considering the timing of its appearance and conversion to peak 2 as discussed above.

Effect of Molybdate on DNA–Sepharose Profiles. In light of the marked stabilization of steroid binding and inhibition of transformation of steroid receptors by sodium molybdate, its effects on the Ah receptor have been investigated by several laboratories. Denison et al. (1986b), Wilhelmsson et al. (1986), and Kester and Gasiewicz (1987) found only a partial protective effect of molybdate on mouse and rat Ah receptor with respect to density gradient profiles and TCDD binding as assayed by HAP; additionally, molybdate provided minor amelioration of KCl-induced destabilization. To follow up on these observations, we examined the effect of 20 mM Na_2MoO_4 in homogenizing and elution buffers on the DNA–Sepharose profiles of hepatic cytosol from several species. All incubations were at 20 °C for 2 h. In general, changes in DNA binding that were attributable to molybdate were not striking and were somewhat species dependent. In rat liver, specific binding retained by DNA was ~60% of control, with a concomitant increase in the flowthrough fractions; that which was retained eluted principally as peak 2, as in control cytosol. The deleterious effects on the DNA profile of 0.3 M KCl during incubation (partial inhibition of transformation) were not ameliorated by molybdate (data not shown). Similarly, in hamster liver homogenized with molybdate, more specific binding eluted in the flowthrough; however, in this case, the loss of affinity for DNA–Sepharose was primarily due to loss of peak 1. A partial loss of specific binding in peak 2 was seen in molybdate-treated guinea pig liver cytosol. In B6 mouse liver, molybdate appeared to diminish peak 2, with little effect on peak 1 (Figure 6). Thus, at least under these conditions, the Ah receptor appears to be more resistant than steroid

Table II: Sedimentation Coefficients (S) of Ah Receptor in Cytosols, Nuclear Extracts, and Peaks Isolated by DNA–Sepharose Chromatography

species		low salt	high salt
rat	cytosol[a]	9.7 ± 0.4[b]	7.1 ± 0.3
	unoccupied	10.4	10.4
	peak 2[a]	7.4	7.0
	nuclear	8.8	6.0
hamster[c]	cytosol	8.1 ± 0.3	6.3 ± 0.2
	peak 1	5.7 ± 0.3	5.4 ± 0.1
	peak 2	6.9 ± 0.5	7.0 ± 0.2
	nuclear	10.4 (6.7)[d]	6.6
guinea pig	cytosol	8.8 ± 0.4	6.9 ± 0.1
	unoccupied	9.8	9.6
	peak 1[e]	4.9	4.6
	peak 2[c]	6.8	6.7
	nuclear	10.6 (6.5)[d]	6.3

[a] Rat cytosol was incubated at 0 °C for 20 h to maximize transformation to peak 2. Insufficient amounts of peak 1 are obtainable from rat for sucrose density gradient analysis. [b] Mean ± SD is indicated for cytosols, n = 3–8. All chromatography experiments were performed at least twice; data for nuclear and unoccupied receptor are from one experiment (two to three determinations). [c] 2 h, 20 °C incubation. [d] Values in parentheses are from nuclear extract subjected to DNA–Sepharose chromatography to obtain the isolated peak 2. [e] 2 h, 0 °C incubation.

Table III: Stokes Radius (R_S) and Calculated Molecular Mass (M_r) of Ah Receptor Forms

species		R_s (nm)	M_r[a]
rat	unoccupied	7.6	314 000
	peak 2	5.1 ± 0.3[b]	147 000
	flowthrough	5.3	162 000
	nuclear extract	5.3[c]	131 000
hamster	peak 1	5.3	118 000
	peak 2	6.0 ± 0.0	173 000
	flowthrough	6.0	166 000
guinea pig	unoccupied	6.8	269 000
	peak 1	4.7 ± 0.0	89 000
	peak 2	6.2 ± 0.3	171 000

[a] Calculated according to the method of Dension et al. (1986a) by using the S values determined on sucrose density gradients prepared in 0.4 M KCl. [b] Where standard deviations are indicated, n = 3–4; otherwise, values are from one or two determinations. [c] The same value was obtained whether or not nuclear extract was previously chromatographed on DNA–Sepharose.

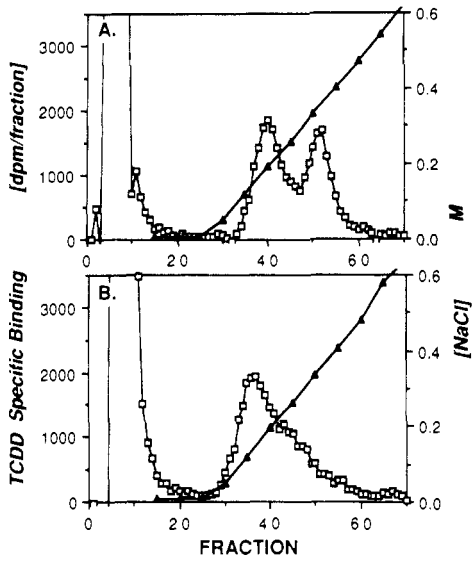

FIGURE 6: Effect of molybdate on the DNA–Sepharose profile of B6 mouse hepatic cytosol. Cytosol (5 mg of protein/mL) was prepared from perfused livers of B6 mice in either HEDG buffer (A) or HEDG plus 20 mM Na_2MoO_4 (B) and incubated with 3 nM TCDD ± TCDF for 2 h at 20 °C. Samples were prepared and chromatographed as described for Figure 1 except that in (B) eluting buffers also contained 20 mM molybdate.

receptors to modification by molybdate; however, the observed effects of molybdate, although small, are qualitatively consistent with its more striking inhibition of steroid receptor transformation to a DNA-binding state.

Sucrose Density Gradient Analysis of Specific Binding Peaks from DNA–Sepharose. Transformation of steroid receptors is sometimes defined in terms of altered sedimentation coefficient on sucrose gradients, which in some cases has been correlated with increased DNA affinity [e.g., Reker et al. (1985), Rowley et al. (1986), and Moudgil and Hurd (1987)]. Since the Ah receptor has frequently been quantified by using sucrose gradients [e.g., Denison et al. (1986a,b) and Wilhelmsson et al. (1986)], it is important to determine the relationship between the different forms of the receptor as detected by DNA–Sepharose and by sucrose density gradients. Additionally, comparison of sedimentation coefficients of the different DNA-binding forms is a further means of characterizing their interrelationships and the changes associated with transformation. For these studies, cytosols from rat, hamster, and guinea pig were incubated with TCDD ± TCDF under conditions that maximized amounts of peak 1 or 2 in each species. Dextran/charcoal-treated cytosols were chromatographed on DNA–Sepharose and peaks were determined by immediately counting aliquots of fractions. The three fractions containing the peak of specific binding in each case were pooled, BSA was added (final concentration 4–5 mg/mL) to minimize adsorptive losses of TCDD·receptor from these dilute solutions, and samples were frozen (−80 °C) for analysis 1–4 days later. Aliquots (400 μL) of these pools were analyzed on sucrose density gradients as described under Experimental Procedures. Portions of the same dextran/charcoal-treated cytosols as had been chromatographed were diluted in HEDG buffer plus NaCl to the same salt concentrations as the pooled peaks from the columns (i.e., ~0.33 M for peak 2 and ~0.15 M for peak 1), and 400 μL of these preparations were run concurrently in each case. Additionally, the sedimentation of unoccupied Ah receptor from rat and guinea pig liver was analyzed by using untreated cytosol as described under Experimental Procedures. The S values calculated from all of these experiments are summarized in Table II.

In each species, sedimentation coefficients measured in TCDD-labeled cytosol shifted to a lower value in high-salt gradients, which has been reported previously [e.g., Okey et al. (1979), Okey (1980), Poellinger et al. (1983), Denison and Wilkinson (1985), Denison et al. (1986a), and Kester and Gasiewicz (1987)] and is presumed to reflect dissociation of protein aggregates or loosely associated subunits. In both hamster and guinea pig, a very low S value was observed for peak 1 isolated from DNA–Sepharose, and this was not affected by 0.4 M KCl (insufficient amounts of peak 1 were found in rat cytosol under any incubation conditions for this analysis). S values for peak 2 isolated by DNA–Sepharose were higher than for peak 1, but also showed little effect of KCl in the gradients. On low-salt gradients, S values for peak 2 were slightly lower than observed in whole cytosol; however, adjustment of rat and guinea pig cytosols to 0.33 M NaCl had no effect on S values determined on low-salt gradients, indicating that the results for peak 2 were not simply a salt effect. [Hamster receptor in whole cytosol adjusted to 0.33 M NaCl was apparently more resistant to reassociation on low-salt gradients, at least when the short centrifugation time possible with vertical tube rotors was used (not shown).] Flowthrough fractions from DNA–Sepharose showed somewhat variable behavior on sucrose gradients, but in general resembled whole cytosol (not shown). Unoccupied receptor from rat and guinea pig sedimented at about 10 S on low-salt gradients, suggesting that it is a larger species than both DNA-binding forms of the liganded receptor (Table II), and it did not dissociate to a smaller form on high-salt gradients.

Nuclear receptor from livers of [^{3}H]TCDD-treated animals sedimented like peak 2 on high-salt gradients. On low-salt gradients, nuclear receptor, unlike peak 2, appeared to be associated with other protein(s) or to undergo aggregation, yielding a higher S value. However, when peak 2 was isolated from these nuclear extracts by DNA–Sepharose chromatography, it sedimented like peak 2 isolated from cytosol. Thus, KCl likely extracts nuclear proteins with which the TCDD·receptor complex remains associated on low-salt sucrose gradients, but which dissociate from the receptor on high-salt gradients and during DNA–Sepharose chromatography.

Gel Filtration Analysis of the Isolated Receptor Forms. Stokes radii, determined on a Superose 6 column with 0.4 M NaCl in the eluting buffer, corroborated the smaller size of receptor in peak 1 compared with peak 2 and the large size of unoccupied receptor (Table III). By use of these data and the S values obtained on 0.4 M KCl sucrose gradients (Table II), the apparent molecular mass (M_r) of each form was calculated according to the method of Denison et al. (1986a) (Table III). Interestingly, the M_rs calculated for peak 1 (89 000 and 118 000 for guinea pig and hamster, respectively) are close to the values reported by Poland and Glover (1987) for photoaffinity-labeled receptors from these species analyzed by denaturing gel electrophoresis, suggesting that both protocols yield a small, monomeric form of the receptor. The values for peak 2, flowthrough fractions, and nuclear extract were all in the range 130 000–170 000, were larger than for peak 1, and were similar to values reported by Prokipcak and Okey (1988) for nuclear and transformed cytosolic (mouse) receptor.

Discussion

Using DNA–Sepharose chromatography, Gasiewicz and Bauman (1987) distinguished three forms of the rat hepatic Ah receptor that differed in affinity for DNA and in surface charge; the distribution among the forms depended on incubation conditions. We have extended that observation by

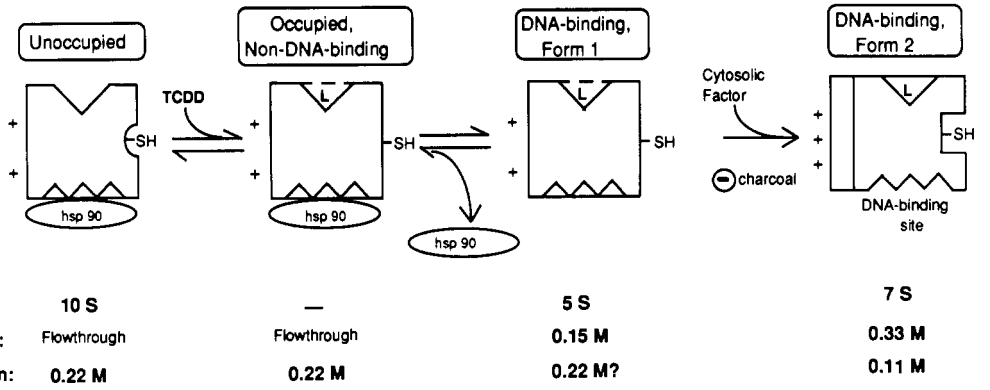

FIGURE 7: Proposed model of Ah receptor processing. Beneath each illustrated form of the receptor is indicated its sedimentation coefficient (high-salt gradients) and the NaCl concentration at which it elutes from DNA- and DEAE-Sepharose columns. "L" indicated in some forms is bound ligand (TCDD). "Cytosolic Factor" represents the additional component(s) in the cytosol necessary for transformation and whose importance was demonstrated in the reconstitution experiments shown in Figures 4 and 5. The identification of the protein associated with the first two forms as hsp90 is hypothesized, on the basis of our results, on published data from Perdew (1988) and Denis et al. (1988a) and on several studies of the interactions of hsp90 with steroid receptors (cf. Discussion). The occupied non-DNA-binding form is not present in all species; although it is illustrated between unoccupied receptor and form 1, it is not necessarily an intermediate conformation (cf. Discussion). The reversibility or irreversibility shown for each conversion is inferred from many indirect observations, principally regarding ligand binding kinetics, but has not been proven.

showing that the same forms are found in liver and in other tissues of guinea pig, hamster, and mouse, that their relative amounts can be manipulated by various modifying factors during incubation, and that they are different conformations of the same protein. Furthermore, after in vivo treatment of all these species with [^{3}H]TCDD, only one of these forms is found in hepatic nuclear extracts; this transformed form of the receptor, which has the highest affinity for DNA–Sepharose, is likely the active form at the genomic level.

Among these species, the two DNA-binding forms showed remarkably similar affinities, as defined by the salt concentrations necessary for their elution (Figures 1 and 2). Variation in DNA–Sepharose profiles among species and tissues was only in the proportion of total TCDD specific binding that bound to DNA and in its distribution between peaks 1 and 2. Other reported studies of DNA binding of the Ah receptor have used DNA–cellulose columns that have generally been eluted isocratically with 0.5 M NaCl (Hannah et al., 1986; Wilhelmsson et al., 1986; Cuthill & Poellinger, 1988). Carlstedt-Duke et al. (1981) also used NaCl gradient elution and observed two peaks of specific binding (at ~0.1 and ~0.27 M), although they evaluated this finding no further. In all cases, retention of TCDD specific binding on DNA was low compared to our results with the same species (rat). This disparity may partially reflect differences between DNA–Sepharose and cellulose. More importantly, we found that the highest retention on DNA was achieved by using cytosol incubation times and temperatures that maximized TCDD binding as assayed by HAP (Kester & Gasiewicz, 1987) and percent transformation; the short incubation at 0–4 °C used in the majority of other studies is insufficient for maximal binding and transformation.

The qualitatively similar behavior of the TCDD·Ah receptor from different species and tissues on DNA–Sepharose suggests that its interaction with total DNA does not contribute significantly to the species and tissue specificity of responsiveness to TCDD. However, it is possible that there are species-dependent differences in more relevant interactions of receptor with specific DNA sequences such as the dioxin responsive elements (Durrin & Whitlock, 1987) and/or with additional nuclear proteins involved in regulating gene expression, neither of which are measured by using bulk DNA linked to Sepharose. Consistent with the overall similarities among receptor molecules, it is also notable that ED50s for several responses to TCDD exposure, unlike LD50s, are comparable among species (Gasiewicz et al., 1986). The quantitative differences in DNA–Sepharose profiles of cytosolic receptor may reflect significant species- and tissue-specific regulation of an active form of the TCDD·receptor complex in vivo. Alternatively, these differences may be due to differing stability under the chromatographic conditions. That in vitro stability varies among species was evident in a comparison of specific binding as measured by HAP, DNA–Sepharose, and dextran/charcoal: in some cases, notably guinea pig and hamster, values on DNA–Sepharose exceeded those from HAP and dextran/charcoal, whereas in rat the reverse was true (data not shown). In hamster, this was found to at least partially reflect the fact that the untransformed receptor (peak 1) does not bind to, or is unstable on, HAP (Rucci & Gasiewicz, 1988). In general, these results do point out that the relative amounts of the receptor forms are very species and tissue specific, at least under in vitro conditions. They may also explain, in part, some reported species differences in physicochemical properties (Poellinger et al., 1983; Denison et al., 1986a), sensitivity to molybdate and salt (Denison et al., 1986a,b), and binding kinetics (Poland et al., 1976; Farrell & Safe, 1987). Note also that the existence of several receptor forms of differing properties and in amounts dependent on incubation conditions makes it difficult to compare molecular parameters such as size and sedimentation with studies in which preparations contain a mixture of these forms.

The observation that after in vivo exposure to [^{3}H]TCDD only peak 2 was present in nuclear extracts in all species examined is strong evidence that this fully transformed receptor is the transcriptionally active conformation. The similarity of sedimentation behavior of receptor from peak 2 and from nuclear extract (Table II) is consistent with this conclusion. Additionally, this nuclear receptor was detectable in [^{3}H]-TCDD-treated D2 mice, corroborating the report by Mason and Okey (1982), who used sucrose density gradient analysis. This finding suggests that the Ah receptor in the D2 mouse functions as in other species, at least in its nuclear association and DNA-binding ability; furthermore, our reconstitution experiments (Figures 4 and 5) verify that D2 cytosol contains the necessary cofactors for transformation.

On the basis of results presented here and elsewhere (Gasiewicz & Bauman, 1987; Henry and Gasiewicz, unpublished data), we propose a working model, outlined in Figure 7, of the different forms of the Ah receptor. Initially, binding of

TCDD to the unoccupied receptor causes a change in the protein that confers on it some affinity for DNA (form 1 = peak 1), although in some species a portion of the ligand-bound receptor lacks DNA affinity (Table I). This process may also expose a sulfhydryl moiety (Henry et al., 1988). It is not clear whether or not a change in overall surface charge occurs: both the unoccupied receptor and cytosol labeled for 2 h at 0 °C elute at the same NaCl concentration from DEAE-Sepharose (Gasiewicz & Bauman, 1987), but since those experiments were performed with rat hepatic cytosol, the labeled cytosol also contained specific binding that appears in the DNA flowthrough and which might confound interpretation of DEAE chromatography. Like the better characterized steroid receptors, the Ah receptor likely contains distinct ligand-binding and DNA-binding sites, since partial proteolysis eliminates DNA binding without affecting ligand binding (Carlstedt-Duke et al., 1981; Wilhelmsson et al., 1986). Presumably, TCDD binding elicits a conformational change that serves to expose the DNA-binding site. Ligand binding and conversion to peak 1 also appears to involve dissociation of some component(s) from the unoccupied receptor, yielding a smaller species (Tables II and III). A possible candidate for this component is the 90-kDa heat shock protein (hsp90), which forms a molybdate-stabilized complex with unoccupied steroid receptors, thereby inhibiting DNA binding of unoccupied steroid receptors; it is released as a dimer from the unoccupied receptor by hormone binding (Pratt, 1987; Denis et al., 1987; Radanyi et al., 1989). Recent reports indicate that hsp90 is also associated with the Ah receptor (Perdew, 1988; Denis et al., 1988a). Indeed, the decrease in M_r between the unoccupied receptor and peak 1 (Table III) is consistent with the loss of a dimer of hsp90. The fact that molybdate inhibits formation of the DNA-binding forms of the Ah receptor, albeit only partially, is also consistent with this conjecture. At present, it is unknown whether or how the interaction of hsp90 with the Ah receptor or the steroid hormone receptors serves a regulatory role in vivo. Although in Figure 7 specific binding that does not bind to DNA is illustrated as a form between the unoccupied receptor and form 1, this material in flowthrough fractions may not represent an obligate intermediate form of the receptor, since it is not detected in all species (Table I). Also, the functional importance of the flowthrough specific binding is not clear; since its behavior on density gradients suggests a mixture or aggregate of components, it is possible that it results from association of the receptor with a component of hsp90 and/or with other molecules such as RNA that may inhibit DNA binding, although this effect is species dependent.

Further incubation of the TCDD-labeled cytosol (or elevated temperature, depending on species) causes a transformation to a form with lower net negative surface charge (Gasiewicz & Bauman, 1987), higher DNA affinity (peak 2), a higher sedimentation coefficient, and a larger Stokes radius. The latter observations suggest that during transformation the receptor associates with another cytosolic component (or perhaps dimerizes), yielding a larger complex. If this component is also associated with the untransformed receptor, it is only a loose interaction, since it is removed by DNA–Sepharose chromatography. Transformation, therefore, may entail stabilization of such a complex. It is only this transformed form that is found in the nucleus and which likely mediates TCDD-induced changes in gene expression. This form also has greater resistance to disruption by sulfhydryl-modifying reagents (Henry et al., 1988) and to dissociation of ligand (Kester, 1987). During review of this paper, a paper by Prokipcak and Okey (1988) appeared which reported that transformation of the Ah receptor in Hepa 1 cells to the nuclear binding form involves a significant change in molecular mass, consistent with our observations. They found that nuclear receptor (6.2 S, M_r = 176 000) is smaller than cytosolic receptor in low ionic strength buffer, but larger than the subunit found in high-salt conditions.

The process of transformation appears to be neither proteolytic nor salt-induced; it does depend on some cytosolic cofactor(s) that can be removed by charcoal. It is likely that the presence of the intermediate form (peak 1) is extremely transient in vivo at 37 °C; for example, nuclear-bound TCDD is significantly elevated within 30 min (earliest time point) of in vivo injection of [^{3}H]TCDD (Greenlee & Poland, 1979). At the lower temperatures used for in vitro incubations, however, we can distinguish a two-step mechanism of receptor processing as defined by changes in behavior on DNA–Sepharose. TCDD·Ah receptor transformation to the high-affinity DNA-binding form occurs quickly even at 20 °C, but longer incubation at 0–4 °C allows the equivalent transformation. It is not clear whether a single mechanism of transformation simply proceeds faster at high temperatures or whether two mechanisms exist, only one of which is temperature dependent.

The rodent Ah receptor and the steroid hormone (particularly glucocorticoid) receptors are structurally and functionally equivalent in many respects, such as the physicochemical properties of the transformed receptors (Wilhelmsson et al., 1986), the requirement of additional cytosolic cofactors for transformation (Figure 4; Grippo et al., 1983; Tienrungroj et al., 1987b; Schmidt et al., 1985), and the fact that ligand binding occurs at 0–4 °C. Indeed, the Ah receptor may ultimately be acknowledged as a member of the steroid receptor superfamily (Evans, 1988). However, the process and regulation of transformation of the receptor·ligand complexes to their nuclear binding forms differ in detail: (1) Glucocorticoid·receptor complexes remain in a non-DNA-binding form unless exposed to further treatment such as 25 °C, gel filtration, or 0.5 M KCl [reviewed by Schmidt and Litwack (1982)]; in molybdate-containing buffers, the non-DNA-binding form is stable even under these conditions. The Ah receptor, on the other hand, apparently does not exist exclusively in a stable ligand-bound form lacking affinity for DNA(–Sepharose); even preparation in the presence of molybdate does not substantially stabilize such a non-DNA-binding form (Figure 6). (2) The partially purified, untransformed glucocorticoid receptor can be converted to its DNA-binding form by heat-stable cytosolic factor(s) (Schmidt et al., 1985; Harmon et al., 1988); cytosolic components that can reconstitute transformation of the isolated untransformed or the charcoal-treated Ah receptor, however, appear to be heat labile (Henry and Gasiewicz, unpublished results). (3) Several papers have suggested that steroid hormone is not necessary for receptor (progesterone, glucocorticoid) transformation to a DNA-binding form to occur, although this effect may be restricted to in vitro conditions and/or purified receptor [Bailly et al., 1986; Becker et al., 1986; Willmann & Beato, 1986; Moudgil & Hurd, 1987; review by Gorski and Hansen (1987)]. In our experiments, the unoccupied Ah receptor had no affinity for DNA–Sepharose, with or without prior heat treatment (1 h at 20 °C), at least as monitored by ligand-binding ability; TCDD binding was apparently an essential step in achieving DNA-binding activity. (4) Recent evidence suggests that ligand binding increases the rate of dissociation of hsp90 from the hormone-binding unit of glu-

cocorticoid and progesterone receptors, and that once dissociated, the receptors have higher affinity for DNA (Pratt, 1987; Howard & Distelhorst, 1988; Denis et al., 1988). Molybdate stabilizes the hsp90·receptor complexes in the non-DNA-binding state. The minimal effectiveness of molybdate toward the Ah receptor suggests that perhaps the association of hsp90 with the TCDD-occupied Ah receptor is more labile than with steroid receptors. However, the unoccupied Ah receptor appears to be stable as a complex, since it retains a high sedimentation coefficient and Stokes radius even in the absence of molybdate and in the presence of KCl (Tables II and III). (5) The fact that two DNA-binding forms of the Ah receptor can be distinguished appears to be unique. However, this must be qualified by noting that DNA binding of glucocorticoid receptors has been evaluated almost exclusively by using batch DNA–cellulose assays or high-salt isocratic elution of DNA–cellulose columns; therefore, it is not known whether analysis of the glucocorticoid receptor under the gradient conditions used here would also reveal two DNA-binding forms.

In summary, Ah receptor in several tissues from rat, B6 mouse, hamster, and guinea pig are qualitatively very similar with respect to their affinity for DNA–Sepharose. Two DNA-binding forms of the TCDD·receptor complex can be distinguished; the lower affinity form is apparently an intermediate conformation of the protein, and in the presence of other cytosolic factor(s) it undergoes transformation to a higher affinity form. Since only the latter form is found in nuclei of TCDD-treated animals, this is likely the biologically active conformation. Even in the relatively TCDD-insensitive D2 mouse, specifically bound TCDD can be detected in the nucleus of treated animals, and its elution from DNA–Sepharose is identical with that of other species. Changes in sedimentation behavior and Stokes radius associated with ligand binding and transformation of the Ah receptor in liver indicate that these processes may involve removal of and association with additional components of the cytosol. Although receptors from different species show some structural variability as determined by hydrodynamic analyses, the interaction of the different receptor forms with DNA–Sepharose is strikingly consistent among species.

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Registry No. TCDD, 1746-01-6.

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A Developmentally Regulated Gene of Trypanosomes Encodes a Homologue of Rat Protein-Disulfide Isomerase and Phosphoinositol-Phospholipase C†,‡

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ABSTRACT: We have isolated and characterized a developmentally regulated gene in *Trypanosoma brucei*, arbitrarily termed BS2. BS2 mRNA is substantially more abundant in bloodstream-form trypanosomes than in procyclic culture forms. Its nucleotide sequence reveals a single contiguous open-reading frame of 497 codons and is predicted to encode a protein of ~55.5 kilodaltons. A search of the NBRF protein data base revealed that within the predicted amino acid sequence are two of the evolutionarily conserved redox sites typified by thioredoxin of bacteria. Of this family of proteins, the recently sequenced rat genes encoding protein-disulfide isomerase (PDI) and form I phosphoinositide-specific phospholipase C (PIPLC) showed homology extending over the length of all three proteins (i.e., between BS2, PDI, and PIPLC). Although this homology includes the acidic C-terminus characteristic of proteins localized to the lumen of the enoplasmic reticulum, the BS2 product is predicted to possess multiple sites for N-linked glycosylation while PDI and PIPLC have none. Possible roles of the BS2 gene product in trypanosome physiology are discussed.

The complex life cycle of pathogenic trypanosomes involves developmental stages in both a mammalian host and the insect vector *Glossina*. Survival and growth in these very different environments demand not only gross morphological changes but also adaptive changes in important biochemical pathways. For example, transition from the bloodstream form of the African *Trypanosoma brucei* to the procyclic form found in the insect involves the rapid shedding of their dense surface coat (Vickerman, 1985; Overath et al., 1983). This developmental switch is also accompanied by a change from utilization of the glycolytic pathway as the principal energy source to activation of a greatly expanded mitochondrial system of oxidative respiration (Vickerman, 1962, 1965; Opperdoes, 1987). These morphologic and metabolic changes are associated with

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‡ The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02865.
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