0026-895X/93/030511-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:511-518

ACCELERATED COMMUNICATION

Role of the Aryl Hydrocarbon Receptor Nuclear Translocator Protein in Aryl Hydrocarbon (Dioxin) Receptor Action

MARKUS R. PROBST,[1] SUZANNE REISZ-PORSZASZ,[1] ROSALLY V. AGBUNAG, MAY S. ONG, and OLIVER HANKINSON

Laboratory of Structural Biology and Molecular Medicine (M.R.P., S.R.-P., R.V.A., M.S.O., O.H.) and the Department of Pathology and Laboratory Medicine (O.H.), School of Medicine, University of California, Los Angeles, California 90024

Received May 18, 1993; Accepted June 21, 1993

SUMMARY

Immunoprecipitation experiments performed on cytosolic extracts of the mouse hepatoma cell line Hepa-1c1c7 (Hepa-1) confirm that the 9-S, unliganded, cytosolic aryl hydrocarbon (Ah) receptor complex contains the 90-kDa heat shock protein and the Ah receptor protein but reveal that it does not contain the Ah receptor nuclear translocator (ARNT) protein. These experiments confirm that the 6-S liganded form of the receptor identified in nuclear extracts of cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) contains the Ah receptor protein and ARNT but not the 90-kDa heat shock protein. The 6-S liganded Ah receptor complex activates transcription of the *CYP1A1* gene via its binding to upstream xenobiotic-responsive elements (XREs). Treatment of cytosolic extracts of Hepa-1 cells with TCDD *in vitro* transforms the Ah receptor complex to the XRE-binding state. No such transformation occurs in a C^- mutant deficient in ARNT activity. When *in vitro* synthesized ARNT was added concomitantly with TCDD to C^- cytosolic extracts, it associated with the Ah receptor and restored Ah receptor-dependent XRE-binding activity to the extracts. Covalent cross-linking experiments in nuclear extracts of Hepa-1 and human LS180 cells treated with TCDD *in vivo* demonstrate that both ARNT and the Ah receptor bind directly to the XRE core sequence.

The Ah receptor[2] binds a variety of environmentally important carcinogens, including polycyclic aromatic hydrocarbons, heterocyclic amines, and halogenated aromatic hydrocarbons such as TCDD and polychlorinated biphenyls (1) (reviewed in Ref. 2). The liganded receptor complex activates transcription of the *CYP1A1* and *CYP1A2* genes and several other genes involved in xenobiotic metabolism (reviewed in Ref. 3). The CYP1A1 and CYP1A2 proteins play important roles in the metabolic activation of polycyclic aromatic hydrocarbons and heterocyclic amines, respectively, to their ultimate carcinogenic metabolites (reviewed in Refs. 4 and 5). The Ah receptor also mediates most, if not all, of the carcinogenic and toxic effects of the halogenated aromatic hydrocarbons, although metabolism of these compounds does not appear to be involved (6).

The location of the unliganded Ah receptor complex has not been fully resolved (7), although the weight of evidence suggests that this moiety is cytoplasmic (8, 9) and it is found in the cytosolic fraction after conventional subcellular fractionation. The composition of the unliganded receptor complex has not been fully determined. The complex sediments at about 9 S in sucrose gradients and is about 280 kDa in size (10). It contains the Ah receptor (90 kDa in Hepa-1 cells and certain mouse strains) (11, 12), HSP90 (13, 14), and perhaps two other proteins (15). After TCDD treatment, the Ah receptor is found in the nuclear fraction, as assessed by conventional subcellular fractionation. Operationally, therefore, transformation of the Ah receptor complex by TCDD results in translocation of the Ah receptor to the nucleus. The nuclear form of the receptor stimulates transcription of the *CYP1A1* gene via interaction

This work was supported by National Cancer Institute Grant CA28868, Department of Energy Contract DE-FC03-87ER 60615, and National Cancer Institute Core Grant CA16042 to the UCLA Jonsson Comprehensive Cancer Center. M.R.P. was supported by a fellowship from the Swiss National Research Foundation and the Basler Freiwillige Akademische Gesellschaft and Fellowship J920910A from the UCLA Jonsson Cancer Center Foundation.

[1] The contributions of the first two authors should be considered equal.

[2] "Ah receptor" refers to the 90-kDa ligand-binding subunit. "Ah receptor complex" refers to any multimeric protein complex containing the Ah receptor.

ABBREVIATIONS: Ah, aryl hydrocarbon; ARNT, aryl hydrocarbon receptor nuclear translocator; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay(s); GST, glutathione-*S*-transferase; HSP90, 90-kDa heat shock protein; PAGE, polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dBrUTP, bromo-dUTP; Hepa-1, Hepa-1c1c7.

with XRE sequences located 5′ to the coding region of the gene (16, 17). The liganded Ah receptor can be extracted from nuclei associated with the 87-kDa ARNT protein (18). This Ah receptor-ARNT heterodimer is about 176 kDa in size and sediments at about 6 S in sucrose gradients (19, 20).

Elferink *et al.* (21) provided evidence that the form of the Ah receptor complex that binds the XRE contains two polypeptides that bind the XRE core sequence directly. One of these polypeptides was identified as the Ah receptor, but the other was not identified. We showed previously that ARNT is found associated with the Ah receptor during binding to the XRE, although those experiments did not allow us to ascertain whether ARNT binds the XRE directly (18). In this paper we investigate whether ARNT binds the XRE directly and therefore corresponds to the second polypeptide detected by Elferink *et al.* (21). This seemed likely for several reasons, including the fact that both ARNT and the Ah receptor contain basic helix-loop-helix motifs (11, 12, 22). These motifs are found in a variety of transcription factors, where they function in both protein dimerization and DNA binding. We also address the question as to whether ARNT is a component of the 9-S unliganded Ah receptor complex.

The Hepa-1 cell line is a widely used model for studying the mechanism of action of the Ah receptor. Mutants of the Hepa-1 line with reduced or undetectable inducibility of CYP1A1 enzyme activity, and defective in Ah receptor function, have been assigned to three complementation groups, probably representing three different genes (reviewed in Ref. 23). In mutants of group C, the cytosolic receptor is present at normal levels as assessed by ligand binding, is of apparently normal size (9 S), and is capable of binding ligand but is unable to translocate to the nucleus after binding ligand (24). C^- mutants are defective in ARNT function, although both of the C^- mutants contain normal levels of ARNT mRNA (22). In certain of the present studies we utilize a C^- mutant to investigate the role of ARNT in the TCDD-dependent transformation of the Ah receptor complex to the XRE-binding state.

Materials and Methods

Chemicals. TCDD was obtained from the National Cancer Institute Chemical Carcinogen Repository. Safety precautions were taken during preparation, handling, and disposal of solutions containing TCDD (17). [^{35}S]Methionine and [^{32}P]dATP were from Amersham and NEN DuPont, respectively. All other chemicals purchased were of the highest quality available.

Antisera. A polyclonal antiserum against the mouse Ah receptor was raised in New Zealand White rabbits using the published amino-terminal peptide sequence (25) coupled to keyhole limpet hemocyanin via an additional cysteine at the amino terminus (Immuno-Dynamics). The antiserum was affinity-purified using the amino-terminal peptide coupled to bovine serum albumin and immobilized on CNBr-activated Sepharose (Pharmacia), according to the manufacturer's instructions. The human ARNT cDNA was digested with *Eco*RI, and the carboxyl-terminal fragment coding for amino acids 399–777 was isolated and cloned into the GST fusion vector pGEX-1λT (Pharmacia). The resulting plasmid, pGEX1λT-ARNT-1.2, was then used to transform *Escherichia coli* JM101 to ampicillin resistance. Colonies were isolated and checked for correct orientation of the insert. Large-scale cultures were induced with 1 mM isopropyl β-D-thiogalactopyranoside at an A_{600} value of 0.9 and were grown for an additional 5 hr at 37°. The fusion protein was purified from inclusion bodies according to the method of Marston (26) and was used to immunize New Zealand White rabbits. Bacterial extracts of JM101 expressing the GST and GST-ARNT-1.2 fusion proteins were prepared and immobilized on CNBr-activated Sepharose. To adsorb all nonspecific antibodies, the polyclonal ARNT antiserum was diluted 1/10 in 10 mM Tris·HCl, pH 7.5, and incubated with immobilized bacterial extract expressing GST only. After centrifugation, the supernatant was incubated with Sepharose beads containing immobilized GST-ARNT-1.2 extract. A column was packed with the Sepharose beads and washed consecutively with 10 mM Tris·HCl, pH 7.5, and with 10 mM Tris·HCl, pH 7.5, containing 0.5 M NaCl. The specific ARNT-1.2 antibodies were eluted with 100 mM glycine, pH 2.5. The antiserum that recognizes the mouse 84-kDa and 86-kDa heat shock proteins (27) was a generous gift of Dr. Steven Ullrich, National Institutes of Health (Bethesda, MD). An IgG fraction of the rabbit antiserum was prepared and used without further purification.

Cell culture and cellular extracts. The mouse hepatoma cell line Hepa-1, the C^- mutant c4, and the human colon adenocarcinoma cell line LS180 were cultured and maintained as described (28). Cells were harvested by the scraping of confluent monolayers into ice-cold phosphate-buffered saline. Cytosolic extracts were made from cells cultured in the absence of TCDD. Nuclear extracts were made from cells that had been treated with TCDD (2 nM for Hepa-1, 10 nM for LS180) in culture for 90 min at 37° before harvesting in ice-cold phosphate-buffered saline. Protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 40 units/ml aprotinin) and 1 mM DTT were added to all cytosolic and nuclear extraction buffers immediately before use.

Cytosolic extracts for EMSA and for UV cross-linking experiments were prepared by the method of Shapiro *et al.* (29). Nuclear extracts for the EMSA and UV cross-linking experiments were prepared from cells that had been suspended in 10 mM HEPES, pH 7.5, for 15 min on ice, centrifuged, resuspended in 1 cell pellet volume of HED (25 mM HEPES, 2 mM EDTA, 1 mM DTT, pH 7.5), and homogenized with a loose-fitting Dounce homogenizer. Cell breakage was monitored under the microscope. After centrifugation (10 min, 14,000 × *g*, 4°), the supernatant (cytosolic fraction) was discarded. The nuclear pellet was resuspended in 1 pellet volume of HED containing 0.4 M KCl (HEDK) and was rocked at 4° for 30 min. After homogenization with a tight fitting pestle, glycerol was added to a final concentration of 20% (v/v). The nuclear fraction was centrifuged at 180,000 × *g* for 30 min at 4°, and the supernatants were then frozen at −80° until further analysis. Nuclear extracts for immunoprecipitation and immunoblotting experiments were prepared as described above except that the HED buffer for swelling the cells and the HEDK nuclear extraction buffer were supplemented with 20 mM sodium molybdate. Nuclear extracts were prepared in the absence of glycerol and were centrifuged at 180,000 × *g* for 120 min rather than 30 min. Also, the nuclear supernatant was dialyzed overnight against 250 volumes of HEDG (HED containing 10% glycerol) with 20 mM Na_2MoO_4 and was then centrifuged for 10 min at 14,000 × *g* at 4°. The cytosolic extracts for the immunoprecipitation and immunoblotting experiments corresponded to the supernatant obtained after Dounce homogenization of these last cells. Protein concentration of all extracts was determined according to the method of Bradford, using bovine serum albumin as standard.

Immunoprecipitation and immunoblotting. Immunoprecipitation of cytosolic and nuclear extracts was performed with a modification of the method described by Poland *et al.* (30). In brief, extracts diluted in HEDG, supplemented with 20 mM Na_2MoO_4, 1% Tergitol NP40 (Sigma), 150 mM NaCl, and 5 mM EGTA, were precleared with Protein A-agarose (Boehringer-Mannheim) on a rocking table for 3 hr at 4°. Affinity-purified antibodies against Ah receptor were added to the supernatant, which was then incubated overnight at 4°. Immuncomplexes were precipitated with Protein A-agarose for 3 hr at 4°, washed five times with the aforementioned buffer, and boiled with SDS-sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 40 mM Tris·HCl, pH 6.8) for 8 min. Supernatants were subjected to SDS-PAGE using a Miniprotean-II gel electrophoresis apparatus (Bio-Rad). For immunoblots, proteins were transferred to nitrocellulose (Hybond-ECL; Amersham) by means of a semi-dry blotting technique. After

blocking with 5% BLOTTO (5%, w/v, nonfat dry milk powder, 0.1% Tween 20 in Tris-buffered saline) containing 4% goat donor serum (Sigma), the membranes were probed with the antibody preparations, appropriately diluted in 5% BLOTTO. Antigen-antibody complexes were detected with a goat anti-rabbit antibody coupled to horseradish peroxidase (Pierce Chemicals), using the enhanced chemiluminescence (ECL) detection system (Amersham). Prestained molecular weight markers (Sigma) were used. Because the staining process significantly alters the molecular weight of the marker proteins, so that the nominal molecular weights provided by the manufacturer are incorrect, the molecular weights of the prestained markers were calibrated by comparing them with a series of nonstained molecular weight markers.

EMSA. Preparation of the ^{32}P-labeled double-stranded synthetic oligonucleotide, which contained base pairs −999 to −979 of the mouse *CYP1A1* gene (encompassing XRE1) and additional nucleotides at both ends, and the EMSA were essentially as described (18). In some experiments EMSA was performed with cytosolic extracts that had been incubated with 10 nM TCDD (added from a 100× stock solution in dimethylsulfoxide) *in vitro* for 3 hr at room temperature. During *in vitro* transformation of the cytosolic extracts of the C$^-$ mutant by TCDD, COS-7 cytosolic extracts transfected with pBM5/NEO-M1-1 or pBM5/NEO or *in vitro* synthesized ARNT protein was added before gel shift analysis, as indicated.

Generation of the ARNT protein. Human ARNT was generated from the ARNT expression vector pBM5/NEO-M1-1 (22), either via transfection into COS-7 cells or via *in vitro* transcription/translation. Transfection of COS-7 cells with pBM5/NEO-M1-1 or pBM5/NEO and labeling with [^{35}S]methionine were performed as described previously (18). Cytosolic extracts of COS-7 cells were prepared as described above for EMSA. For *in vitro* transcription/translation assays, the TNT T7-coupled reticulocyte lysate system containing RNase inhibitor (RNAsin) was programmed with pBM5/NEO-M1-1 according to the manufacturer's instructions (Promega).

UV cross-linking. The primer 5′-TGAGCTCG-3′ was annealed to the oligonucleotide 5′-TCCGGCTCTTCTCACGCAACTCCGAGCTC A-3′, and dBrUTP (Sigma) instead of dTTP was used for second-strand synthesis with DNA polymerase I (Klenow fragment; New England Biolabs). Thus, three dBrUTPs (shown in bold) were incorporated in the second strand, as follows: 5′-TGAGCTCGGAGUUGC GUGAAGAAGAGCCGGA-3′. Nuclear extracts (100 μg of protein), diluted in 25 mM HEPES, pH 7.5, containing 1 mM DTT, 1 mM EDTA, and 10% glycerol, were incubated with 2.5 μg of poly(dI·dC) (Pharmacia) for 20 min at room temperature and then incubated with the labeled XRE for another 20 min. UV cross-linking was performed by irradiation with a Photodyne transilluminator, emitting predominantly at λ 302 nm, for 30 min. Either the incubation mixture was irradiated directly, or it was subjected to nondenaturing PAGE and the portion of the gel containing the receptor-XRE complex was irradiated, and the receptor-XRE complex then electroeluted from the gel overnight in 20 mM HEPES, 20 mM Tris·HCl, 1 mM EDTA, pH 8.0. Irradiated material was boiled in SDS-sample buffer for 3 min to disrupt protein-protein complexes and all protein-XRE complexes except those in which protein and DNA were covalently cross-linked. The electroeluted protein-XRE complexes were precipitated with acetone and subjected to denaturing SDS-PAGE (7.5%). To the complexes irradiated in solution, affinity-purified Ah receptor or ARNT antibodies or the corresponding preimmune IgG were added in some cases, as indicated in the text, and incubated overnight at 4°. Immuncomplexes were precipitated for 2 hr with Protein A-agarose (which had been previously equilibrated in 10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.3 M NaCl, 10 μg/ml bovine serum albumin), washed three times in the same buffer, and then subjected to SDS-PAGE (7.5%). The dried gel was exposed at −80° to Kodak X-OMAT AR X-ray film.

Results

Characterization of affinity-purified antibodies to ARNT and the Ah receptor. Affinity-purified Ah receptor and ARNT antibodies were prepared as described in Materials and Methods. When cytosolic extracts prepared from Hepa-1 or human LS180 cells grown in the absence of TCDD (i.e., "uninduced cytosols") were probed on immunoblots with the antibodies to the carboxyl-terminal half of human ARNT, a single protein, migrating at about 95 kDa, was detected in both extracts (Fig. 1A, *lanes 1* and *5*). This is close to the known size of 87 kDa for human ARNT (22). This protein was also detected in nuclear extracts prepared from cells of both strains that had been grown with TCDD (i.e., "induced nuclei") (Fig. 1A, *lanes 2* and *6*). The amount of the protein was greatly diminished in uninduced cytosol and induced nuclei of the C$^-$ mutant, which is deficient in ARNT activity (Fig. 1A, *lanes 3* and *4*). Considerably more of the protein was detected in extracts prepared from strain vT{2} (data not shown). vT{2} is a derivative of the C$^-$ mutant transfected with the human ARNT cDNA expression vector pBM5/NEO-M1-1, which is known to overexpress ARNT mRNA (22, 31). These results demonstrate that the ARNT antibodies are monospecific for ARNT in both cytosolic and nuclear fractions.

Antibodies generated to the 19-amino acid amino-terminal peptide of the mouse Ah receptor detected a single protein in

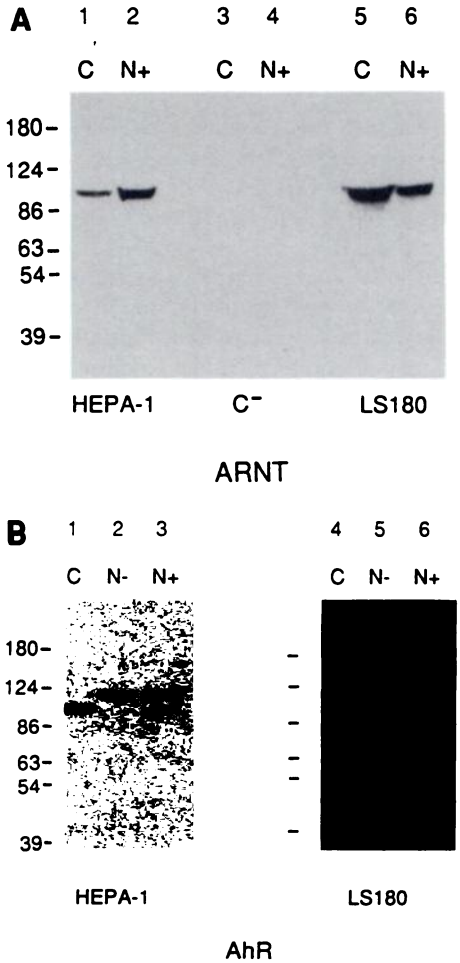

Fig. 1. Characterization of affinity-purified polyclonal Ah receptor and ARNT antibodies. Protein (100 μg) from uninduced cytosolic (*C*), induced nuclear (*N+*), or uninduced nuclear (*N−*) extracts of Hepa-1, C$^-$ mutant, and LS180 cells were subjected to SDS-PAGE, transferred to nitrocellulose, and then probed with ARNT antibodies (A) or Ah receptor antibodies (B). The positions of the molecular weight markers (recalibrated as indicated in Materials and Methods) are indicated on the *left*.

uninduced cytosols from Hepa-1 cells and LS180 cells, of about 95 kDa and 110 kDa, respectively (Fig. 1B, *lanes 1* and *4*). These are close to the expected sizes for the Ah receptor in Hepa-1 and LS180 cells, i.e., 90 kDa (11, 12) and 110 kDa (32), respectively. Thus, the Ah receptor antibodies specifically detect the corresponding protein in cytosols of these strains. The antibodies also uniquely detected the Ah receptor in induced nuclei of LS180 cells (Fig. 1B, *lane 6*). The receptor was not found in uninduced nuclei of LS180 cells (Fig. 1B, *lane 5*), consistent with the known behavior of the receptor. In induced nuclei of Hepa-1 cells, the antibodies detected a protein of about 110 kDa, as well as the approximately 95-kDa Ah receptor. The larger protein was also detected in uninduced nuclei of Hepa-1 cells (Fig. 1B, *lane 2*) and therefore does not correspond to a precursor or derivative of the Ah receptor. The larger protein (like the Ah receptor) was not detected if the antibodies were preabsorbed with the Ah receptor peptide used to generate the antibodies or if preimmune serum was used (data not shown), demonstrating that the larger protein represents a species that cross-reacts with the Ah receptor antibodies. It should be noted that ARNT and the Ah receptor from Hepa-1 cells migrated at indistinguishable rates on SDS-PAGE.

Demonstrated that ARNT is not a component of the cytosolic 9-S Ah receptor complex. We were unable to immunoprecipitate the unoccupied cytosolic receptor complex with ARNT antibodies. We therefore used another approach to ascertain whether this complex contains ARNT. Induced nuclei and uninduced cytosols of Hepa-1 cells were treated with antibodies to the Ah receptor, and the resulting immunoprecipitates were subjected to SDS-PAGE and then probed on Western blots with the ARNT antibodies. ARNT was not detected in immunoprecipitates from uninduced cytosolic extracts (Fig. 2, *lane 3*), whereas it was detected in immunoprecipitates from induced nuclear extracts, as expected (Fig. 2, *lane 4*). [Because the Ah receptor antibodies immunoprecipitate, in addition to the Ah receptor, another protein from nuclear extracts of Hepa-1 cells (Fig. 2), another formal interpretation of this last finding is that ARNT is associated with the latter protein, rather than the Ah receptor, in nuclei.] To exclude the possibility that the Ah receptor antibodies are unable to immunoprecipitate the 9-S cytosolic complex, we also probed the precipitated cytosolic and nuclear complexes with Ah receptor antibodies. The Ah receptor was detected in the cytosolic extracts (Fig. 2, *lane 1*) as well as the nuclear extracts (Fig. 2, *lane 2*), demonstrating that the Ah receptor antibodies are able to precipitate both forms of the receptor. Both precipitates were also probed with HSP90 antibodies. Whereas HSP90 was identified in the uninduced cytosolic form of the receptor (Fig. 2, *lane 5*), it was not detected in the nuclear form (Fig. 2, *lane 6*). These experiments therefore demonstrate that ARNT is not a component of the untransformed cytosolic Ah receptor complex. They also indicate that HSP90 is not a component of the transformed nuclear receptor complex.

Association of exogenous ARNT with the Ah receptor after TCDD treatment of cytosol *in vitro*. The C^- Hepa-1 cell mutant c4 is defective in ARNT function. In this cell line, the 9-S Ah receptor complex is present, but it cannot be transformed *in vivo* to the XRE-binding state by TCDD (24). *In vitro* synthesized and ^{35}S-labeled ARNT protein was concomitantly added with TCDD to a cytosolic extract of the C^- mutant, which was then incubated for 3 hr at room temperature. When these extracts were immunoprecipitated with Ah receptor antibodies, coprecipitation of labeled ARNT was observed (Fig. 3, *lane 1*). In cytosol not treated with TCDD, the ARNT protein was barely detectable after immunoprecipitation with Ah receptor antibodies (Fig. 3, *lane 2*). A very faint signal was also observed when preimmune serum was used (Fig. 3, *lane 5*). The faint signal was most likely due to a nonspecific interaction of labeled ARNT protein with IgG, because no precipitation of ARNT protein occurred in the absence of antibodies (Fig. 3, *lanes 3* and *4*). These experiments demonstrate that exogenous ARNT associates with the Ah receptor in cytosolic extracts after the extracts are treated with TCDD.

Restoration of XRE binding in C^- extracts by addition of ARNT. An induced nuclear extract of Hepa-1 cells was incubated with the ^{32}P-labeled double-stranded XRE-containing oligonucleotide and then subjected to nondenaturing PAGE. The position of the band corresponding to the Ah receptor-XRE complex is indicated in Fig. 4 (*lane 1*, *arrow*). This band was not detected when a 100-fold excess of unlabeled XRE was added to the incubation mixture (Fig. 4, *lane 2*). It was also not detected with uninduced nuclear extracts of Hepa-1, with induced nuclear extracts of the C^- mutant (22), or with uninduced Hepa-1 cytosol (Fig. 4, *lane 3*). When a cytosolic extract of Hepa-1 cells was treated with TCDD *in vitro*, the Ah receptor-XRE complex was produced (Fig. 4, *lane 4*). However, this complex was not generated when a cytosolic extract of the C^- mutant was treated with TCDD *in vitro* (Fig. 4, *lane 6*). When extracts prepared from COS-7 cells that had been transfected with the human ARNT cDNA expression vector pBM5/NEO-M1-1 or the parental plasmid pBM5/NEO were added to C^- cytosol and the mixture was treated with TCDD, the Ah receptor-dependent band was observed with the extract containing ARNT (Fig. 4, *lane 7*) but not with the extract prepared from COS-7 cells containing the parental plasmid (Fig. 4, *lane 8*). Similarly, when increasing amounts of a rabbit reticulocyte

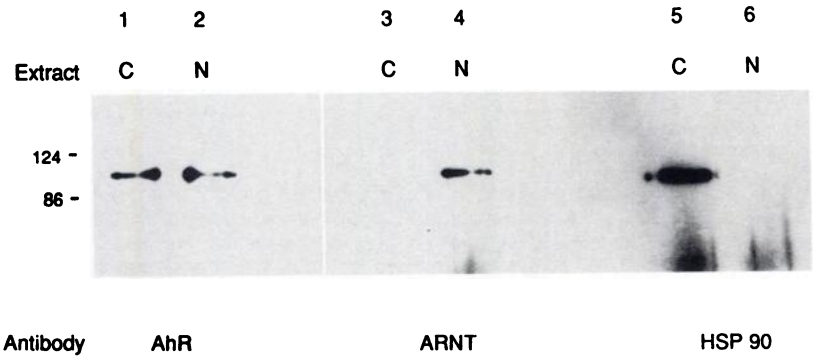

Fig. 2. ARNT is not a component of the untransformed cytosolic Ah receptor complex. Uninduced cytosolic (*C*) or induced nuclear (*N*) extracts from Hepa-1 cells (200 μg of protein each) were immunoprecipitated with affinity-purified Ah receptor antibodies, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with Ah receptor, ARNT, and HSP90 antibodies. The molecular weight markers are indicated on the *left*. Only the range above 65 kDa is shown, to exclude the bands corresponding to IgG light and heavy chains.

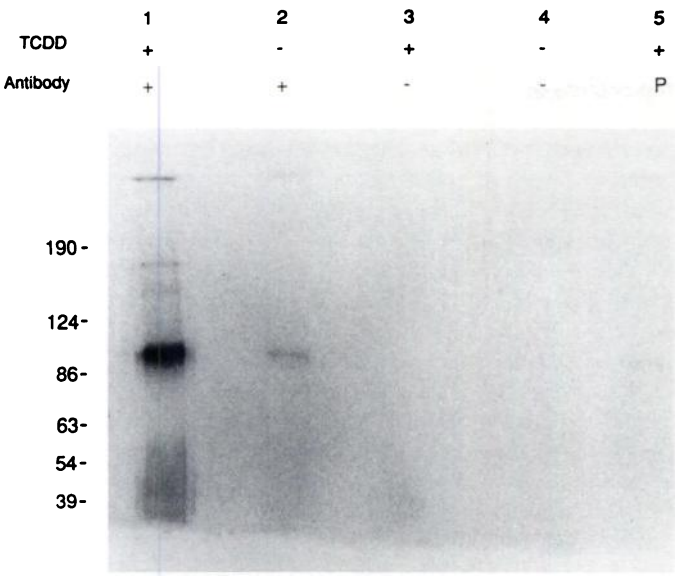

Fig. 3. Exogenous ARNT can associate with the Ah receptor in TCDD-treated cytosol. Uninduced cytosol from the C⁻ mutant (150 μg of protein) was incubated at room temperature with 8 μl of rabbit reticulocyte lysate containing *in vitro* transcribed, translated, and ^{35}S-labeled ARNT, in the presence (+) or absence (−) of 10 nM TCDD. After treatment with Ah receptor antibodies (+), corresponding preimmune IgG (*P*), or no antibodies (−), precipitates were run on SDS-PAGE and subjected to autoradiography.

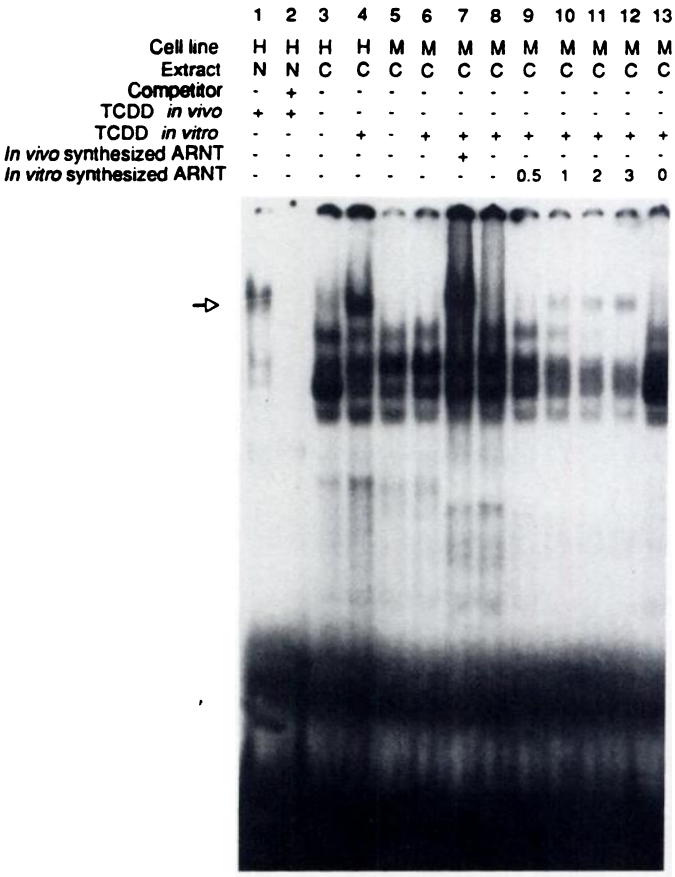

Fig. 4. Restoration of Ah receptor-dependent XRE binding to the C⁻ mutant. EMSA of Hepa-1 and C⁻ mutant cells were performed using 100 μg of protein for cytosolic extracts and 5 μg of protein for nuclear extracts. *H*, Hepa-1; *M*, C⁻ mutant; *C*, uninduced cytosolic extract; *N*, induced nuclear extract. *Arrow on the left*, position of the Ah receptor-XRE complex. *Lane 2*, a 100-fold excess of unlabeled double-stranded oligonucleotide was included. *Lanes 7* and *8*, 10 μg of protein from cytosolic extracts of COS-7 cells, transfected with pBM5/NEO-M1-1 or pBM5/NEO, respectively, were added. *Lanes 9-12*, increasing amounts (0.5–3 μl) of rabbit reticulocyte lysate programmed with ARNT were added. *Lane 13*, 3 μl of unprogrammed lysate.

lysate containing *in vitro* synthesized ARNT were added to the C⁻ cytosol, they resulted in the generation of increasing amounts of the protein-XRE complex (Fig. 4, *lanes 9-12*). The protein-XRE complex was not generated when the reticulocyte lysate was programmed with the parental plasmid pBM5/NEO (Fig. 4, *lane 13*). Thus, in the C⁻ cytosol, TCDD-dependent binding of the Ah receptor complex to the XRE can be restored by addition of ARNT protein *in vitro*.

UV cross-linking of the Ah receptor to the dBrUTP-substituted XRE. Cell extracts were incubated with a ^{32}P-labeled, double-stranded oligonucleotide containing three dBrUTPs in the core sequence of the XRE. The extracts were irradiated with UV to cross-link protein molecules to the XRE core sequence. They were then boiled in SDS-sample buffer to disrupt protein-protein interactions and noncovalent protein-DNA interactions, run on SDS-PAGE, and exposed to X-ray film (Fig. 5). We did not treat the extracts with DNase before electrophoresis, because the electrophoretic mobility of Ah receptor-dependent protein-XRE complexes is not recognizably affected by such treatment (21). Several cross-linked protein-DNA complexes were observed when an induced nuclear extract from Hepa-1 cells was used (Fig. 5, *lane 1*). The most prominent of these had apparent molecular sizes of 44, 46, 67, 75, 95, and 140 kDa. The presence of a 100-fold excess of unlabeled XRE abolished all of the bands (Fig. 5, *lane 2*). The protein-DNA complex at approximately 95 kDa was not observed when either a nuclear or cytosolic extract of uninduced Hepa-1 cells was used (Fig. 5, *lanes 4* and *5*, respectively). When uninduced cytosolic extracts from Hepa-1 or C⁻ mutant cells were transformed with TCDD *in vitro* and subsequently UV cross-linked, the 95-kDa band was observed in Hepa-1 cells (Fig. 5, *lane 6*) but not in the C⁻ mutant (Fig. 5, *lane 7*). However, when a cytosolic extract of COS-7 cells transiently transfected with pBM5/NEO-M1-1 was added simultaneously with TCDD during *in vitro* transformation of cytosol of the C⁻ mutant, the 95-kDa cross-linked complex was generated (Fig. 5, *lane 8*). The 95-kDa complex was not seen when a cytosolic extract of COS-7 cells transfected with the parental plasmid pBM5/NEO was used (data not shown). Generation of the 95-kDa band is therefore dependent upon TCDD, a functional Ah receptor, and ARNT and must therefore correspond to a protein-XRE complex (or complexes) between the Ah receptor complex and the XRE. TCDD did not enhance formation of any of the other protein-DNA complexes in either nuclei or cytosols of Hepa-1 cells (compare Fig. 5, *lane 1* with *lane 4* and *lane 6* with *lane 5*, respectively). The latter complexes were also observed using cytosols from the C⁻ mutant (compare Fig. 5, *lane 7* with *lane 5*). Formation of these complexes is therefore not dependent upon TCDD, a functional Ah receptor, or ARNT. These complexes may correspond to constitutive XRE-binding proteins, such as those previously detected in Hepa-1 extracts (33), or to other proteins that bind DNA sequences other than the XRE core sequence that are present in the double-stranded oligonucleotide used for the EMSA.

When the Ah receptor-XRE complex from TCDD-treated Hepa-1 cells was cross-linked by UV directly in a nondenatu-

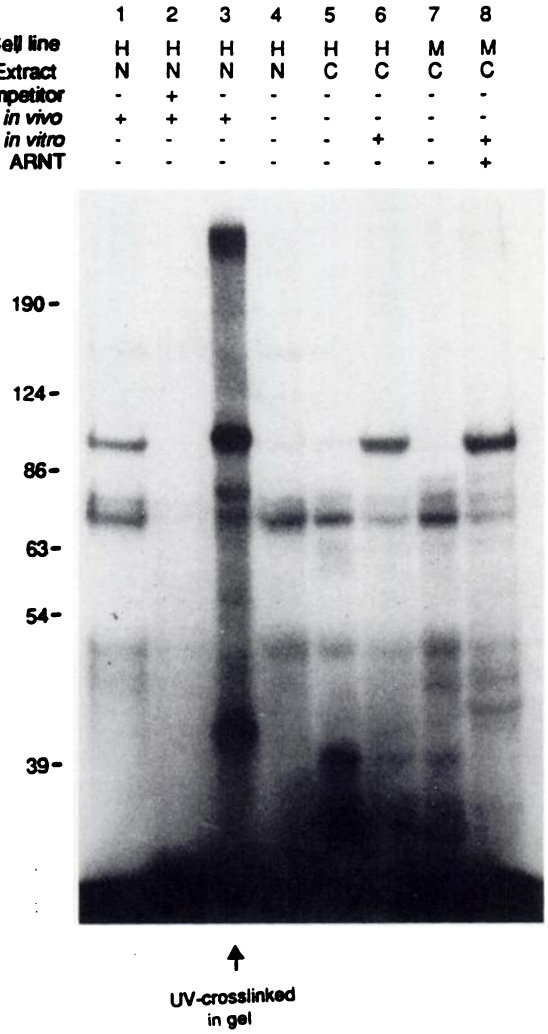

Fig. 5. UV cross-linking of the nuclear Ah receptor complex to the dBrUTP-substituted XRE. Nuclear (*N*) extracts were prepared from Hepa-1 cells (*H*) or C^- mutant cells (*M*) grown in the presence (+) or absence (−) of 2 nM TCDD for 90 min. Cytosolic extracts (*C*) were incubated with (+) or without (−) 10 nM TCDD *in vitro*. The extracts were then incubated with the dBrUTP-containing and ^{32}P-labeled double-stranded oligonucleotide, encompassing XRE1. Proteins were cross-linked to the oligonucleotide in solution, except for *lane 3*, where the Ah receptor-XRE complex was cross-linked directly in the gel after nondenaturing gel electrophoresis and then eluted from an appropriate slice of the gel. *Lane 7*, cytosolic extract of the C^- mutant was cross-linked in the absence of TCDD. *Lane 8*, cytosol was incubated with an extract prepared from COS-7 cells transfected with pBM5/NEO-M1-1 in the presence of TCDD.

ring polyacrylamide gel, electroeluted, and run under denaturing conditions, the 95-kDa complex and certain of the other protein-DNA complexes were observed. Furthermore, two additional bands at about 40 kDa and 220 kDa were also detected. These last bands were also seen on occasion when cross-linking was performed in solution, particularly when longer times of irradiation were used (data not shown). The 220-kDa band could represent a complex in which both XRE-binding components of the Ah receptor are cross-linked to the same XRE molecule (see below), as suggested by Elferink *et al.* (21). The 40-kDa band could represent an additional XRE-binding component of the Ah receptor that is cross-linked only under more intense irradiation conditions or a degradation product of either ARNT or the Ah receptor.

Demonstration that ARNT and the Ah receptor both bind the XRE core sequence directly. Elferink *et al.* (21) found that, after TCDD activation *in vitro*, the Ah receptor complex from rat liver cytosol contained two proteins, of similar but distinct apparent molecular weights, that could be cross-linked to the XRE core sequence and resolved by subsequent SDS-PAGE. One of these proteins was shown to be the Ah receptor; the other was not identified. As described above, however, we detected only one Ah receptor-dependent band (at about 95 kDa) in analogous experiments with Hepa-1 nuclear extracts when cross-linking was performed in solution. We investigated the composition of this band using the ARNT and Ah receptor antibodies. UV cross-linking to the dBrUTP-substituted and ^{32}P-labeled double-stranded XRE oligonucleotide was carried out in solution using nuclear extracts prepared from Hepa-1 cells that had been cultured in the presence of TCDD. The cross-linked extracts were boiled in SDS-sample buffer (to disrupt noncovalent protein-protein interactions), diluted to 0.4% SDS, and then treated with the ARNT and Ah receptor antibodies. Both the ARNT and Ah receptor antibodies precipitated a cross-linked protein-XRE complex of about 95 kDa from Hepa-1 nuclear extracts (Fig. 6, *lanes 3* and *6*, respectively). In the supernatants obtained after immunoprecipitation, the same bands were detected as in the extract not treated with antibodies, except that the intensity of the protein-XRE band at 95 kDa was diminished (compare Fig. 6, *lanes 2* and *5* with *lane 1*). (Note that equivalent amounts of material were loaded in Fig. 6, *lanes 1*, *2*, and *5*, but that 3-fold greater amounts of extract were used to obtain the immunoprecipitates loaded in Fig. 6, *lanes 3* and *6*.) The corresponding preimmune antibody preparations did not precipitate protein-XRE complexes from the Hepa-1 extracts (Fig. 6, *lanes 4* and *7*). These results indicate that the 95-kDa band detected in Hepa-1 extracts contains two different cross-linked complexes, corresponding to the ARNT and Ah receptor proteins individually cross-linked to the core region of the XRE, which were not resolved during electrophoresis. This is consistent with the fact that these two proteins in Hepa-1 cells are both about 90 kDa and cannot easily be resolved by SDS-PAGE (see Fig. 1). Because the Ah receptor antibodies detected another protein in addition to the Ah receptor in nuclear extracts of Hepa-1 cells (albeit of 110 kDa), we also carried out UV cross-linking and immunoprecipitation experiments using nuclear extracts of human LS180 cells, in which the Ah receptor antibodies detect only the Ah receptor (see Fig. 1). The LS180 cell line has the added advantage that it possesses ARNT and Ah receptor proteins that are sufficiently different in size from one another that they can be resolved by SDS-PAGE. The ARNT antibodies precipitated an approximately 95-kDa protein-XRE cross-linked complex from nuclear extracts prepared from LS180 cells grown with TCDD (Fig. 6, *lane 10*). This is consistent with the known size of the human ARNT protein (22) (see Fig. 1). The Ah receptor antibodies precipitated a cross-linked protein-XRE complex of about 110 kDa (Fig. 6, *lane 13*), which is consistent with the size of the Ah receptor in LS180 cells (32) (see Fig. 1). The corresponding preimmune antibody preparations did not precipitate any protein-XRE complexes from LS180 nuclear extracts (Fig. 6, *lanes 11* and *14*). These experiments therefore demonstrate that both ARNT and the Ah receptor bind the XRE directly.

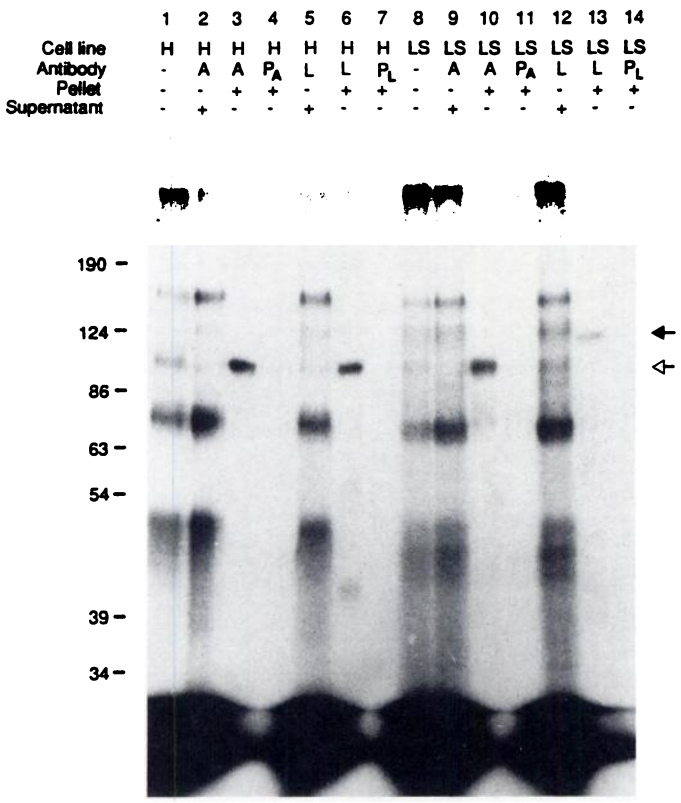

Fig. 6. ARNT and the Ah receptor both interact directly with the XRE core sequence. The dBrUTP-substituted and ^{32}P-labeled double-stranded XRE oligonucleotide was cross-linked in solution with nuclear extracts prepared from Hepa-1 (*H*) or LS180 (*LS*) cells that had been grown with 2 nM or 10 nM TCDD, respectively. The extracts were boiled for 3 min in SDS-sample buffer and diluted, and then ARNT (*A*) or Ah receptor (*L*) antibodies or IgG fractions from the corresponding preimmune sera (P_A and P_L, respectively) were added, as indicated. The resulting immunoprecipitates, or one third of the supernatant from each immunoprecipitation, were then subjected to SDS-PAGE. *Lanes 1* and *8*, cross-linked material, in amounts equivalent to those in the corresponding supernatants, was subjected to SDS-PAGE directly, without immunoprecipitation. Closed arrow, position of the immunoprecipated Ah receptor from LS180 cells. Open arrow, position of immunoprecipitated ARNT from LS180 cells and immunoprecipitated Ah receptor and ARNT from Hepa-1 cells.

Discussion

The size of the untransformed cytosolic Ah receptor complex has been estimated to be about 280 kDa by gel filtration and sucrose gradient analysis (10) and about 340 kDa by chemical cross-linking and SDS-PAGE (15). In the latter case, evidence for subunits of about 97, 96, 88, and 46 kDa was obtained. Because the Ah receptor, HSP90, and ARNT are all about 90 kDa, one possibility is that the unliganded untransformed Ah receptor complex contains one molecule of each of these polypeptides. However, our results show that the immunoprecipitated unliganded receptor complex does not contain ARNT, and they are therefore incompatible with this idea. Furthermore, our analysis of the immunoprecipitated nuclear Ah receptor complex confirms that this complex does contain ARNT. Thus, ARNT must associate with the Ah receptor only after TCDD triggers release of the Ah receptor from the 9-S cytosolic receptor complex. Our demonstration that exogenously added ARNT can associate with the Ah receptor after TCDD treatment of cytosol *in vitro* supports this last proposal. This proposal is also consistent with a model for Ah receptor transformation put forward by Gasiewicz and co-workers (20). Based on physico-chemical analysis, those investigators suggested that TCDD causes release of the free Ah receptor from the 9-S cytosolic form of the receptor and that the free Ah receptor then associates with a cytosolic protein to generate the high affinity DNA-binding form of the receptor (20). We identify this cytosolic factor as being ARNT. Interestingly, in the studies of Gasiewicz and co-workers (20), the cytosolic factor did not appear to be required for TCDD-dependent release of the Ah receptor from the 9-S complex. This therefore suggests that ARNT does not actively participate in the process whereby TCDD triggers dissociation of the 9-S complex.

We and others have observed that after ligand binding *in vivo* the receptor can be found in the nucleus of Hepa-1 cells as a homogeneous species of about 6 S (18, 19). However, Wilhelmsson *et al.* (34) and Perdew (35) detected the 9-S as well as the 6-S species in nuclei of Hepa-1 cells treated with TCDD in culture. When we treated induced nuclei with Ah receptor antibodies, HSP90 did not coimmunoprecipitate with the Ah receptor. Our results therefore do not support the notion that the large 9-S form of the receptor can translocate to the nucleus after binding ligand. However, the difference between the result obtained in our experiment and those obtained by Wilhelmsson *et al.* (34) and Perdew (35) may relate to differences in extraction procedures or to other conditions used in the different experiments, and the aforementioned issue should be considered to remain unresolved.

We previously demonstrated that the 6-S Ah receptor complex extracted from nuclei of Hepa-1 cells grown with TCDD is a heterodimer consisting of the Ah receptor and ARNT. Furthermore, we showed that the XRE-binding form of the receptor contains both proteins (18). However, these experiments did not allow us to determine whether ARNT binds directly to the XRE or associates with the XRE indirectly, by "piggy-backing" on the Ah receptor. Elferink *et al.* (21) provided evidence that the XRE-binding form of the Sprague-Dawley rat Ah receptor complex contains two different proteins that bind the XRE directly. The molecular sizes of these two proteins were determined by SDS-PAGE to be 110 kDa and 100 kDa, and the smaller was identified, by labeling with a photoaffinity ligand, as the Ah receptor. If, as is likely, the rat receptor behaves similarly to the mouse receptor, then the other protein detected by Elferink *et al.* (21) must be ARNT.

Under more intense UV irradiation conditions we observed two additional TCDD-dependent protein-XRE complexes, of about 40 and 220 kDa. The 40-kDa protein could represent a novel XRE-binding protein or a degradation product of either ARNT or the Ah receptor that was generated during processing of the sample. Elferink *et al.* (21) observed a species of about 220 kDa in their cross-linking experiments and showed that this species contained the Ah receptor. They provided evidence that the 220-kDa species corresponds to a complex in which one molecule of the Ah receptor and one molecule of the other XRE-associated protein that they identified are both cross-linked to the same XRE-containing double-stranded oligonucleotide molecule (21). It is therefore likely that the 220-kDa cross-linked complex that we observed represents a complex between the XRE, Ah receptor, and ARNT (and perhaps also the 40-kDa protein).

Based on our results, we provide the following working model

for the mechanism whereby the Ah receptor activates transcription of the *CYP1A1* gene. 1) Binding of the 9-S Ah receptor complex with TCDD or other agonist ligands results in dissociation of HSP90 and possibly other proteins and release of the Ah receptor. 2) The free Ah receptor heterodimerizes with ARNT in the cytosol. 3) The heterodimer translocates into the nucleus. 4) Both ARNT and the Ah receptor bind directly to the core sequences of XREs located 5′ to the coding sequence of the *CYP1A1* gene. 5) Transcription of the *CYP1A1* gene is thereby activated.

In summary, we show that ARNT is not a component of the unliganded cytosolic 9-S Ah receptor complex, and we demonstrate that both subunits of the nuclear Ah receptor complex bind directly to the XRE core sequence. Finally, we have demonstrated that addition of *in vitro* synthesized ARNT protein along with TCDD to cytosolic extracts of the C^- mutant results in association of the ARNT protein with the Ah receptor and its participation in binding to the XRE. These last results are similar to those recently reported by Poellinger and coworkers (36). These results substantiate the working model presented above and also represent a significant step towards reconstituting Ah receptor activity *in vitro*.

Acknowledgments

We thank Dr. Stephen Ullrich for providing us with the antiserum against the murine 84-kDa and 86-kDa heat shock proteins. We also thank Steve Bacsi for his help.

References

1. Kleman, M. I., E. Overvik, M. G. F. Mason, and J.-A. Gustafsson. *In vitro* activation of the dioxin receptor to a DNA-binding form by food-borne heterocyclic amines. *Carcinogenesis (Lond.)* **13**:1619–1624 (1992).
2. Safe, S. H. Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* **26**:371–399 (1986).
3. Whitlock, J. P. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30**:251–277 (1990).
4. Gonzalez, F. J. The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40**:243–288 (1989).
5. Guengerich, F. P. Characterization of human microsomal cytochrome P-450 enzymes. *Annu. Rev. Pharmacol. Toxicol.* **29**:241–264 (1989).
6. Landers, J. P., and N. J. Bunce. The Ah receptor and the mechanism of dioxin toxicity. *Biochem. J.* **276**:273–287 (1991).
7. Whitlock, J. P., and D. R. Galeazzi. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin receptors in wild-type and variant mouse hepatoma cells. *J. Biol. Chem.* **259**:980–985 (1984).
8. Denison, M. S., P. A. Harper, and A. B. Okey. Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: codistribution of unoccupied receptor with cytosolic marker enzymes during fractionation of mouse liver, rat liver and cultured Hepa-1c1 cells. *Eur. J. Biochem.* **155**:223–229 (1986).
9. Gudas, J. M., S. Karenlampi, and O. Hankinson. Intracellular localization of the Ah receptor. *J. Cell. Physiol.* **128**:441–448 (1986).
10. Denison, M. S., L. M. Vella, and A. B. Okey. Hepatic Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: partial stabilization by molybdate. *J. Biol. Chem.* **261**:3987–3995 (1986).
11. Ema, M., N. Watanabe, Y. Chujoh, N. Matsushita, O. Gotoh, Y. Funae, and Y. Fujii-Kuriyama. cDNA cloning and structure of a mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184**:246–253 (1992).
12. Burbach, C. M., A. Poland, and C. A. Bradfield. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89**:8185–8189 (1992).
13. Perdew, G. H. Association of the Ah receptor with the 90-kDa heat shock protein. *J. Biol. Chem.* **263**:13802–13805 (1988).
14. Denis, M., S. Cuthill, A.-C. Wikstroem, L. Poellinger, and J.-A. Gustafsson. Association of the dioxin receptor with the M_r 90,000 heat shock protein. *Biochem. Biophys. Res. Commun.* **155**:801–807 (1988).
15. Perdew, G. H. 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).
16. Fujisawa-Sehara, A., K. Sogawa, M. Yamane, and Y. Fujii-Kuriyama. Characterization of xenobiotic responsive elements upstream from the drug metabolizing cytochrome P450c gene: a similarity to glucocorticoid response elements. *Nucleic Acids Res.* **15**:4179–4191 (1987).
17. Watson, A. J., and O. Hankinson. Dioxin and Ah receptor-dependent protein binding to xenobiotic responsive elements and G-rich DNA studied by *in vivo* footprinting. *J. Biol. Chem.* **266**:6874–6878 (1992).
18. Reyes, H., S. Reisz-Porszasz, and O. Hankinson. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science (Washington D. C.)* **256**:1193–1195 (1992).
19. Prokipcak, R. D., and A. B. Okey. Physicochemical characterization of the nuclear Ah receptor from mouse hepatoma cells exposed in culture to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch. Biochem. Biophys.* **267**:811–828 (1988).
20. Henry, E. C., G. Rucci, and T. A. Gasiewicz. Characterization of multiple forms of the Ah receptor: comparison of species and tissues. *Biochemistry* **28**:6430–6440 (1989).
21. Elferink, C. J., T. A. Gasciewicz, and J. P. Whitlock. Protein-DNA interactions at a dioxin-responsive enhancer. *J. Biol. Chem.* **265**:20708–20712 (1990).
22. Hoffman, E. C., H. Reyes, F.-F. Chu, F. Sander, L. H. Conley, B. A. Brooks, and O. Hankinson. Cloning of a factor required for the activity of the Ah (dioxin) receptor. *Science (Washington D. C.)* **252**:954–958 (1991).
23. Hankinson, O., B. A. Brooks, K. I. Weir-Brown, E. C. Hoffman, B. S. Johnson, J. Nanthur, H. Reyes, and A. J. Watson. Genetic and molecular analysis of the Ah receptor and of CYP1A1 gene expression. *Biochimie (Paris)* **73**:61–66 (1991).
24. Legraverend, C., R. R. Hannah, H. J. Eisen, I. S. Owens, D. W. Nebert, and O. Hankinson. Regulatory gene product of the *Ah* locus. *J. Biol. Chem.* **257**:6402–6407 (1982).
25. Bradfield, C. A., E. Glover, and A. Poland. Purification and N-terminal amino acid sequence of the Ah receptor from the C57BL/6J mouse. *Mol. Pharmacol.* **39**:13–19 (1991); erratum in *Mol. Pharmacol.* **39**:435 (1991).
26. Marston, F. A. O. The purification of eucaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240**:1–12 (1986).
27. Ullrich, S. J., E. A. Robinson, L. W. Law, M. Willingham, and E. Appella. A mouse tumor-specific transplantation antigen is a heat shock-related protein. *Proc. Natl. Acad. Sci. USA* **83**:3121–3125 (1986).
28. Hankinson, O. Selections for and against cells possessing cytochrome P450IA1-dependent aryl hydrocarbon hydroxylase activity. *Methods Enzymol.* **206**:381–400 (1991).
29. Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller. A high-efficiency HeLa cell nuclear transcription extract. *DNA* **7**:47–55 (1988).
30. Poland, A., E. Glover, and C. A. Bradfield. Characterization of polyclonal antibodies to the Ah receptor prepared by immunization with a synthetic peptide hapten. *Mol. Pharmacol.* **39**:20–26 (1991).
31. Reyes, H. Croning of a subunit of the Ah (dioxin) receptor. Ph.D. thesis, University of California, Los Angeles (1991).
32. Harper, P. A., R. D. Prokipcak, L. E. Bush, C. L. Golas, and A. B. Okey. Detection and characterization of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the human colon adenocarcinoma cell line LS180. *Arch. Biochem. Biophys.* **290**:27–36 (1991).
33. Saatciouglou, F., D. J. Perry, D. S. Pasco, and J. B. Fagan. Multiple DNA-binding factors interact with overlapping specificities at the aryl hydrocarbon response element of the cytochrome P450IA1 gene. *Mol. Cell. Biol.* **10**:6408–6416 (1990).
34. Wilhelmsson, A., S. Cuthill, M. Denis, A.-C. Wikstroem, J.-A. Gustafsson, and L. Poellinger. The specific DNA binding activity of the dioxin receptor is modulated by the 90 kD heat shock protein. *EMBO J.* **9**:69–76 (1990).
35. Perdew, G. H. Comparison of the nuclear and cytosolic form of the Ah receptor from Hepa 1c1c7 cells: charge heterogeneity and ATP binding properties. *Arch. Biochem. Biophys.* **291**:284–290 (1991).
36. Whitelaw, M., I. Pongratz, A. Wilhelmsson, J.-A. Gustafsson, and L. Poellinger. Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. *Mol. Cell. Biol.* **13**:2504–2514 (1993).

Send reprint requests to: Oliver Hankinson, Laboratory of Structural Biology and Molecular Medicine, University of California, Los Angeles, 900 Veteran Avenue, Los Angeles, CA 90024.