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Orientation of the Heterodimeric Aryl Hydrocarbon (Dioxin) Receptor Complex on Its Asymmetric DNA Recognition Sequence

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SUMMARY

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-transformed aryl hydrocarbon receptor (AHR) complex binds to xenobiotic-responsive element (XRE) sequences in the 5′ flanking region of the *CYP1A1* gene, resulting in initiation of transcription. Both components of the transformed AHR complex [the ligand-binding AHR monomer and the AHR nuclear translocator (ARNT)] directly contact the XRE. These proteins belong to a novel subclass of basic helix-loop-helix transcription factors. The binding sites of AHR and ARNT on the asymmetric XRE were determined using nuclear extracts of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated Hepa-1c1c7 cells and a panel of double-stranded oligonucleotides containing XRE1 of the *CYP1A1* gene (5′-TTGCGTGAGAA-3′), in which all combinations of three, two, or one of the thymines indicated were substituted by the photoreactive thymine analog 5-bromodeoxyuracil. Covalent cross-linking analysis and immunoprecipitation with antibodies specific for AHR or ARNT demonstrated that ARNT directly contacts the 3′-most thymine position, that AHR directly contacts the second thymine position, and that neither protein contacts the 5′-most thymine position. The thymine position contacted by ARNT lies within a three-nucleotide sequence (5′-GTG-3′) identical to a half-site of an E-box element (5′-CACGTG-3′) that is recognized by a number of other basic helix-loop-helix transcription factors. AHR binds to a portion of the XRE that does not resemble an E-box. Additional experiments demonstrated that neither protein loops over to contact residues located beyond the other's binding site.

The AHR binds a variety of environmentally important carcinogens, including TCDD and benzo(*a*)pyrene, and mediates carcinogenesis by these compounds, either completely or in part (reviewed in Refs. 1 and 2). The unliganded AHR (approximately 90 kDa in C57BL/6 mice) is located in the cytoplasm as part of a complex[1] of about 280 kDa that also contains one or two molecules of the 90-kDa heat shock protein and perhaps other proteins (3, 4). Ligand binding leads to "transformation" of the AHR complex, whereby AHR dissociates from the 280-kDa complex and then associates with the 87-kDa ARNT protein (5–7). After transformation of the AHR complex, induction of CYP1A1 and several other proteins involved in xenobiotic metabolism or growth control occurs in responsive tissues (reviewed in Refs. 2 and 8). In the case of CYP1A1, induction results principally, or even exclusively, from an increase in the rate of transcriptional initiation of the *CYP1A1* gene (9, 10). Transcriptional activation of the *CYP1A1* gene occurs as the result of binding of the transformed AHR complex to several copies of short sequences, termed XREs or dioxin-responsive elements, in the 5′ flanking region of the gene. Both AHR and ARNT bind directly to the XRE sequence (7). Although the transformed

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[1] AHR complex refers to any multimeric protein complex containing the AHR monomer.

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix-loop-helix; dBrU, 5′-bromodeoxyuracil; EMSA, electrophoretic mobility shift assay; Hepa-1, Hepa-1c1c7; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element; PAS, period/aryl hydrocarbon receptor nuclear translocator/aryl hydrocarbon receptor/single-minded.

AHR complex is probably a dimer of AHR and ARNT, the presence of other very small components has not been rigorously ruled out (5).

The AHR and ARNT proteins each contain bHLH motifs near their amino termini (11–13). The basic regions of AHR and ARNT are responsible for XRE binding, and the helix-loop-helix regions are required for heterodimerization between the two proteins (14).[2] AHR and ARNT, however, differ from all or most other bHLH-containing transcription factors in several respects. (i) They both contain an approximately 300-amino acid region of sequence similarity, termed the PAS region. The PAS regions contribute to the establishment of heterodimerization between the two proteins (14).[2] (ii) Activation of the AHR complex requires ligand. (iii) The XRE sequence differs from the E-box sequence, 5′-CANNTG-3′, which is the recognition sequence for nearly all other bHLH-containing transcription factors.

A consensus XRE sequence that can confer ligand-inducible expression on a linked reporter gene has been identified as 5′-T/GNGCGTGA/CG/CA-3′ (15, 16). The consensus XRE sequence for binding of the transformed AHR complex *in vitro* is less restrictive and has been identified as 5′-CGTGA/CG/C/TA/T-3′.[3] The four core nucleotides (5′-CGTG-3′) are absolutely required for binding, whereas substitutions at other positions only reduce binding affinity by up to about 8-fold (15–17). Methylation interference and methylation protection analyses have demonstrated that all guanine residues in the 4-base pair core and also a guanine residue immediately 5′ to the core (which was present in all XREs examined) contact the transformed AHR complex *in vitro* (18–20). The same guanine residues have also been shown to contact the transformed AHR complex *in vivo* (21, 22).

The fact that the XRE sequence is asymmetrical indicates that ARNT and AHR bind different nucleotides in this sequence. The present study was undertaken to determine the orientation of the AHR-ARNT heterodimer on the XRE sequence. To identify the binding positions of the two known subunits of the transformed AHR complex, nuclear extracts from Hepa-1 mouse hepatoma cells treated with TCDD in culture were used in conjunction with a panel of double-stranded synthetic XREs bearing dBrU substitutions in place of thymine residues within and flanking the *in vitro* consensus XRE sequence. UV light treatment of dBrU-substituted DNA molecules produces a highly reactive uracilyl radical, which covalently binds any organic material in direct van der Waals contact (23). Using this approach in conjunction with antibodies to AHR and ARNT, specific thymines of the asymmetrical XRE were found to be in direct contact with the AHR and ARNT subunits of the transformed AHR complex.

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 (24). [^{32}P]dATP was purchased from NEN DuPont. All other chemicals were of the highest quality available.

Cell culture and cellular extracts. Hepa-1 cells (25) were cultured and maintained in Eagle minimum essential medium (α modification) with 10% fetal calf serum, in a 5% CO_2 incubator at 37° (26). Nuclear extracts were prepared essentially as described previously (7). Briefly, approximately 1×10^7 cells in culture were treated with 2×10^{-9} M TCDD for 1.5 hr. The cells were then harvested by scraping into ice-cold phosphate-buffered saline. All subsequent steps were performed on ice. After washing in phosphate-buffered saline and swelling in 10 mM HEPES, pH 7.5, the cells were homogenized with a Dounce homogenizer in HED buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, pH 8.0, 1 mM dithiothreitol) containing protease inhibitors (200 μM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 40 units/ml aprotinin). The nuclei were separated from cytoplasm by centrifugation and were then lysed by addition of KCl to a final concentration of 0.4 M. Glycerol was added to the nuclear fraction to 20%, and ultracentrifugation was used to pellet membranes and associated structures. The protein concentration of the supernatant was determined by the method of Bradford, using bovine serum albumin as a standard. The extract was snap-frozen in liquid nitrogen and stored at −80°.

Oligonucleotide synthesis. Oligonucleotides were either purchased from Integrated DNA Technologies or synthesized on an Applied Biosystems PCRMate model 391 oligonucleotide synthesizer, using the manufacturer's instructions and reagents. dBrU-containing oligonucleotides were synthesized by using dimethoxytrityl-5-bromodeoxyuracil-β-cyanoethyl phosphoramidite (Peninsula Labs). All dBrU-containing oligonucleotides were handled in darkness.

EMSAs. The XRE DNA sequence used for these studies corresponded to base pairs −999 to −979 of the mouse *CYP1A1* gene (encompassing XRE1) with additional nucleotides at both ends, as shown in Table 1 (5). Double-stranded XRE-containing oligonucleotides were synthesized using complementary 30-mer and 18-mer DNA oligonucleotides, which could be annealed and subsequently filled in using DNA polymerase I (Klenow fragment; New England Biolabs) and [α-^{32}P]dATP (specific activity, 6000 Ci/mmol). After radiolabeling, all XREs were passed over two Chromaspin-10 columns (Clonetech) and a single Centrex, 0.45-μm, nitrocellulose spin column (Schleicher & Schuell). EMSAs were performed with nuclear extracts as described previously (5, 7). Briefly, nuclear extract (5 μg of protein) was diluted in 25 mM HEPES, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, pH 8.0, and 10% glycerol, before preincubation with 2.5 μg of poly(dI·dC) at room temperature for 20 min. Subsequently, 15,000–20,000 cpm of radiolabeled double-stranded oligonucleotide were added for 20 min at room temperature. The reactions were then resolved by 4.6% nondenaturing PAGE, followed by autoradiography and analysis by laser densitometry.

Covalent cross-linking. Each covalent cross-linking reaction contained Hepa-1 nuclear extract (300 μg of protein) from cells treated in culture with TCDD. The extract was diluted in 25 mM HEPES, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, pH 8.0, and 10% glycerol, before preincubation with 2.5 μg of poly(dI·dC) at room temperature for 20 min. Subsequently, 300,000–500,000 cpm of purified, radiolabeled, double-stranded oligonucleotide were added for an additional 20 min at room temperature. Covalent cross-linking was performed on ice by irradiation for 30 min with a Fotodyne transilluminator, emitting predominantly at 302 nm, at a distance of 5 cm. UV-irradiated material was boiled in SDS sample buffer (100 mM Tris·HCl, pH 6.8, 2% glycerol, 2% SDS, 1% 2-mercaptoethanol) for 3 min, to disrupt protein-protein complexes and all protein-XRE complexes except those in which protein and DNA were covalently cross-linked.

Antisera and immunoprecipitation. Specifically cross-linked proteins were identified by immunoprecipitation using antisera specific for either ARNT or the AHR protein, followed by SDS-PAGE to resolve the immunoprecipitated/cross-linked species. IgG fractions of polyclonal antibodies specific for ARNT and AHR were used for immunoprecipitation of covalently cross-linked protein-XRE complexes, essentially as described previously (7). Briefly, after UV treatment and boiling in SDS sample buffer, samples were diluted to final concentrations of 35 mM HEPES, pH 7.6, 1.2 mM EDTA, pH 8.0,

[2] B. N. Fukunaga, M. R. Probst, S. Reisz-Porszasz, and O. Hankinson, unpublished observations.

[3] All references to 'consensus XRE' refer to this sequence.

TABLE 1
dBrU-substituted XREs tested

Name	Normal and substituted XRE sequences[a]	Position(s) substituted
	32 1 || |	
XRE	5′-TGAGCTCGGAGTTGCGTGAGAAGAGCCGGA-3′	None
X1	5′-TGAGCTCGGAGUUGCGUGAGAAGAGCCGGA-3′	1, 2, and 3
X2	5′-TGAGCTCGGAGUUGCGTGAGAAGAGCCGGA-3′	2 and 3
X3	5′-TGAGCTCGGAGTUGCGUGAGAAGAGCCGGA-3′	1 and 2
X4	5′-TGAGCTCGGAGUTGCGUGAGAAGAGCCGGA-3′	1 and 3
X5	5′-TGAGCTCGGAGTTGCGUGAGAAGAGCCGGA-3′	1
X6	5′-TGAGCTCGGAGTUGCGTGAGAAGAGCCGGA-3′	2
X7	5′-TGAGCTCGGAGUTGCGTGAGAAGAGCCGGA-3′	3
X8	5′-TGAGCTCGGAGTTGCGTGAGAAGAGCCGGA-3′ * ** *	3′ flanking region
X9	5′-TGAGCTCGGAATTGCGTGAGAAGAGCCGGA-3′ **	5′ flanking region

[a] All XREs were double-stranded, and only the upper strand is shown. U, dBrU substitution in place of thymine. *, dBrU substitution in the opposite strand.

10% glycerol, 200 nM NaCl, and 0.1% Nonidet P-40. Approximately 30 μg of the appropriate antiserum or preimmune serum were then added and the mixture was incubated at 4° for 1 hr, with rocking. Protein A-Sepharose was then added and the incubation was continued for an additional 1 hr. After centrifugation, the resultant specific immune pellets and approximately one third of the supernatant fluid were boiled separately in SDS sample buffer before 7.5% SDS-PAGE and autoradiography, followed by laser densitometry.

Results

Substituted XRE sequences tested. The DNA binding specificities of the AHR and ARNT subunits of the nuclear form of the AHR complex were determined using dBrU-substituted XRE sequences to test all combinations of single, double, and triple substitutions of three thymines located within the central region of XRE1 of the *CYP1A1* gene. Additionally, two XREs, X8 and X9, with dBrU substitutions flanking either side of this region were tested to determine whether thymine residues in these positions play a role in protein-DNA contact (Table 1).

Covalent cross-linking of the AHR complex to XREs with two dBrU substitutions. To analyze the binding specificity of the AHR and ARNT subunits of the AHR complex with respect to the XRE sequence, XREs with double thymine substitutions within the central region of XRE1 were tested by both EMSA and covalent cross-linking analysis. It was found by EMSA that all three doubly dBrU-substituted XREs, i.e., X2, X3, and X4, reproducibly formed complexes with TCDD-treated nuclear extracts and there was no difference in the amounts or rates of migration of the AHR-XRE complexes, compared with unsubstituted XRE1 or the XRE substituted in three positions (positions 1, 2, and 3), X1 (data not shown). With X1, we confirmed that either the AHR- or ARNT-specific antibody specifically immunoprecipitated an approximately 90-kDa protein-XRE complex after covalent cross-linking (Ref. 7 and Fig. 1). With the substituted XRE X2, in which thymine positions 2 and 3 were replaced by dBrU, only the AHR-specific antibody was capable of immunoprecipitating a 90-kDa protein-XRE complex. Using substituted X3, in which thymine positions 1 and 2 were substituted with dBrU, 90-kDa protein-XRE complexes immunoprecipitable by both the AHR- and ARNT-specific antibodies were obtained. Use of X4, in which positions 1 and 3 were substituted, resulted in a 90-kDa protein-XRE complex immunoprecipitable by ARNT-specific antibody only (Fig. 1). Preimmune sera were used in parallel with immune AHR- or ARNT-specific antisera, and in all cases no protein-XRE complexes were detected (data not shown). These results indicated that position 1 cross-linked the ARNT subunit, whereas the AHR subunit was cross-linked to position 2.

Covalent cross-linking of the AHR complex to XREs with one dBrU substitution. To verify the binding positions observed with the XREs substituted with dBrU at two positions, additional XREs (X5, X6, and X7) (Table 1) were synthesized with the same three thymine residues individually replaced by dBrU. Using the same protocol of binding, covalent cross-linking, and immunoprecipitation, we tested whether a single dBrU substitution was sufficient for covalent cross-linking of each of the two subunits.

First, each singly substituted XRE was tested by EMSA. It was found that each of these XREs, X5, X6, and X7, was as efficient as the triply substituted XRE, X1, in binding the AHR complex (Fig. 2A). A second, faster migrating, band, which is unrelated to the transformed AHR complex, was observed in EMSAs using X5, X6, and X7 (Fig. 2A). Covalent cross-linking with X5, in which the position 1 core thymine was replaced with dBrU, produced a 90-kDa protein-XRE complex that could be immunoprecipitated by the ARNT-specific antibody but not by the AHR-specific antibody (Fig. 2B). Covalent cross-linking with X6, in which the thymine at position 2 was substituted with dBrU, resulted in a 90-kDa protein-XRE complex that was immunoprecipitated by the AHR-specific antibody but not by the ARNT-specific antibody. X7, substituted at position 3 with dBrU, failed to be covalently cross-linked with either the AHR or ARNT subunit, although it bound the AHR-ARNT complex efficiently, as analyzed by EMSA. Preimmune sera were used in parallel with immune AHR- or ARNT-specific antisera, and in all cases no protein-XRE complexes were detected. These results were consistent with those obtained with the doubly substituted XREs, and they confirmed that ARNT directly contacted the XRE at the position 1 thymine, that AHR directly contacted the XRE at the position 2 thymine, and that the position 3 thymine did not appear to contact either subunit of the AHR complex.

Covalent cross-linking of the AHR complex to XREs with flanking dBrU substitutions. To test whether either AHR or ARNT contacts regions of the XRE flanking the segment containing the three thymines analyzed above, XREs were prepared in which thymines flanking either side

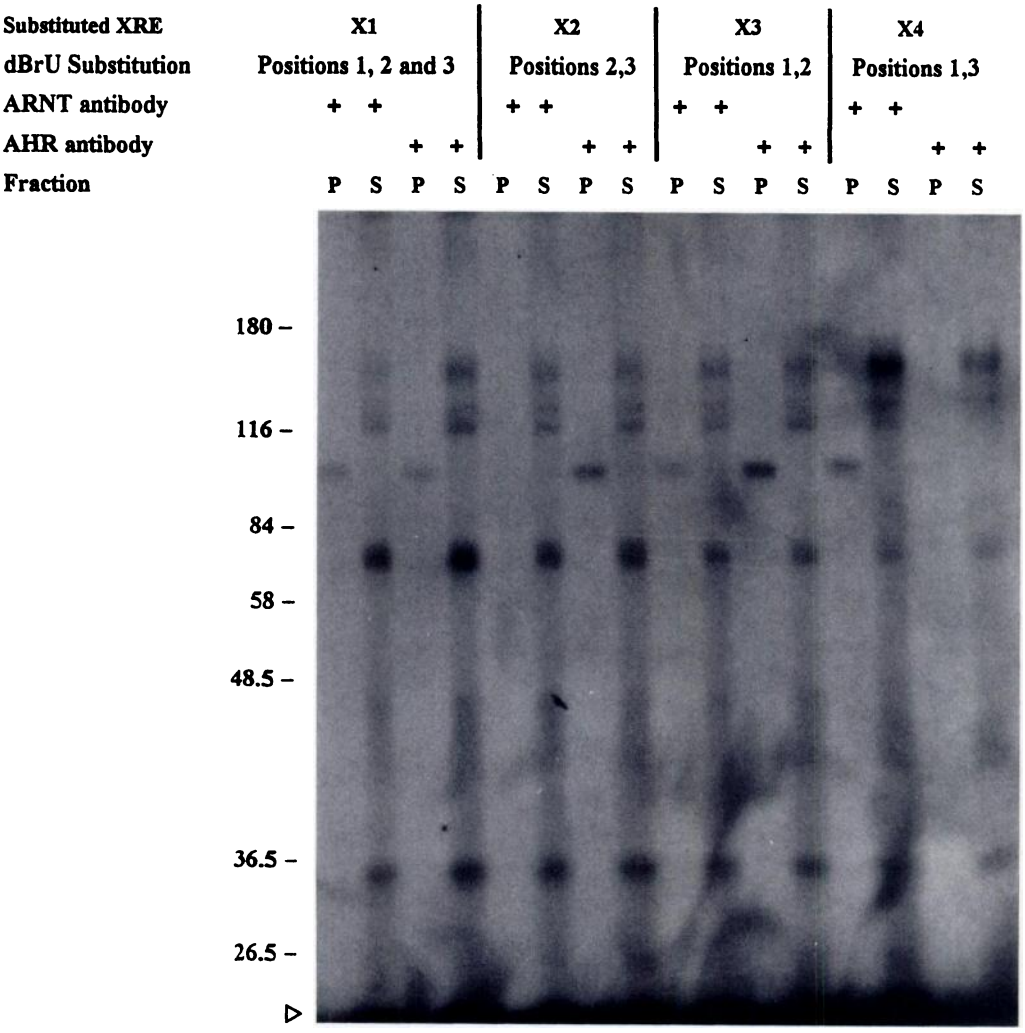

Fig. 1. Covalent cross-linking of the AHR complex to XREs with two dBrU substitutions. Triply dBrU-substituted (X1) and doubly dBrU-substituted (X2, X3, and X4), ^{32}P-labeled, double-stranded XRE oligonucleotides were covalently cross-linked in solution with nuclear extract (300 μg of protein) prepared from Hepa-1 cells grown with 2×10^{-9} M TCDD. After boiling with SDS sample buffer, ARNT- or AHR-specific antibodies were added (as indicated). The immunoprecipitate (*P*) or one third of the supernatant (*S*) from each immunoprecipitation was then subjected to SDS-PAGE and autoradiography. *Open arrowhead*, position of free ^{32}P-labeled XRE. *Numbers to the left*, molecular mass standards (in kDa).

of position 1 or 3 were replaced by dBrU. The substituted XRE X8 was prepared with dBrU incorporated into the lower XRE strand in place of the four thymines most 3′ proximal to the thymine at position 1. Only a single thymine is found in close proximity 5′ to thymine position 3 in XRE1, and this thymine is in the lower strand. This thymine was replaced with dBrU and an additional dBrU was introduced into the same strand immediately 5′ of this position, producing sequence X9, containing two dBrUs (Table 1). The AHR complex bound X8 as efficiently as it bound the triply substituted XRE X1 (Fig. 3, *left*). After covalent cross-linking, only a small amount of a 90-kDa protein-XRE complex was immunoprecipitable by the ARNT-specific antibody (Fig. 3, *right*), representing 9% of the amount observed for the triply substituted XRE X1 (as determined by laser densitometry). No complex was immunoprecipitable with the AHR-specific antibody using X8. Preimmune sera did not immunoprecipitate the protein-X8 complex (data not shown). Covalent cross-linking analysis with the XRE X9 yielded no detectable protein-XRE complexes after immunoprecipitation with either AHR- or ARNT-specific antibody, even though the transformed AHR complex bound X9 with high efficiency, as determined by EMSA (data not shown). These results indicated some degree of contact between the ARNT protein and thymines 3′ (as illustrated in Table 1) to the consensus XRE sequence. Despite efficient XRE-AHR complex formation, as determined by EMSA, the dBrU substitutions within XRE X9 failed to demonstrate proximity to either AHR or ARNT.

Discussion

Different bHLH proteins may bind different forms of the E-box sequence, with the central two nucleotides playing a primary role in discriminating between different bHLH proteins. However, some degree of specificity is also provided by up to three nucleotides flanking both sides of the E-box (27–31). E-boxes can be considered to contain two half-sites, with each bHLH monomer apparently binding nucleotides of only one half-site (32–34). The basic region of ARNT contains a particular arginine consensus residue that, in other bHLH proteins, dictates binding to a particular subclass of the E-box element, 5′-CACGTG-3′ (32, 35, 36). Depending upon its exact boundaries, the basic region of AHR may also contain the appropriate arginine residue. The four 3′-most nucleotides of the E-box sequence described above are identical to the four core nucleotides of the XRE. In the present work we have shown that ARNT binds to the (dBrU-substituted) thymine residue present in the XRE core and, therefore, to the side of the XRE resembling the E-box described above. AHR binds to part of the XRE sequence not resembling an E-box. This is consistent with the observation that the basic region of ARNT conforms well to the consensus for

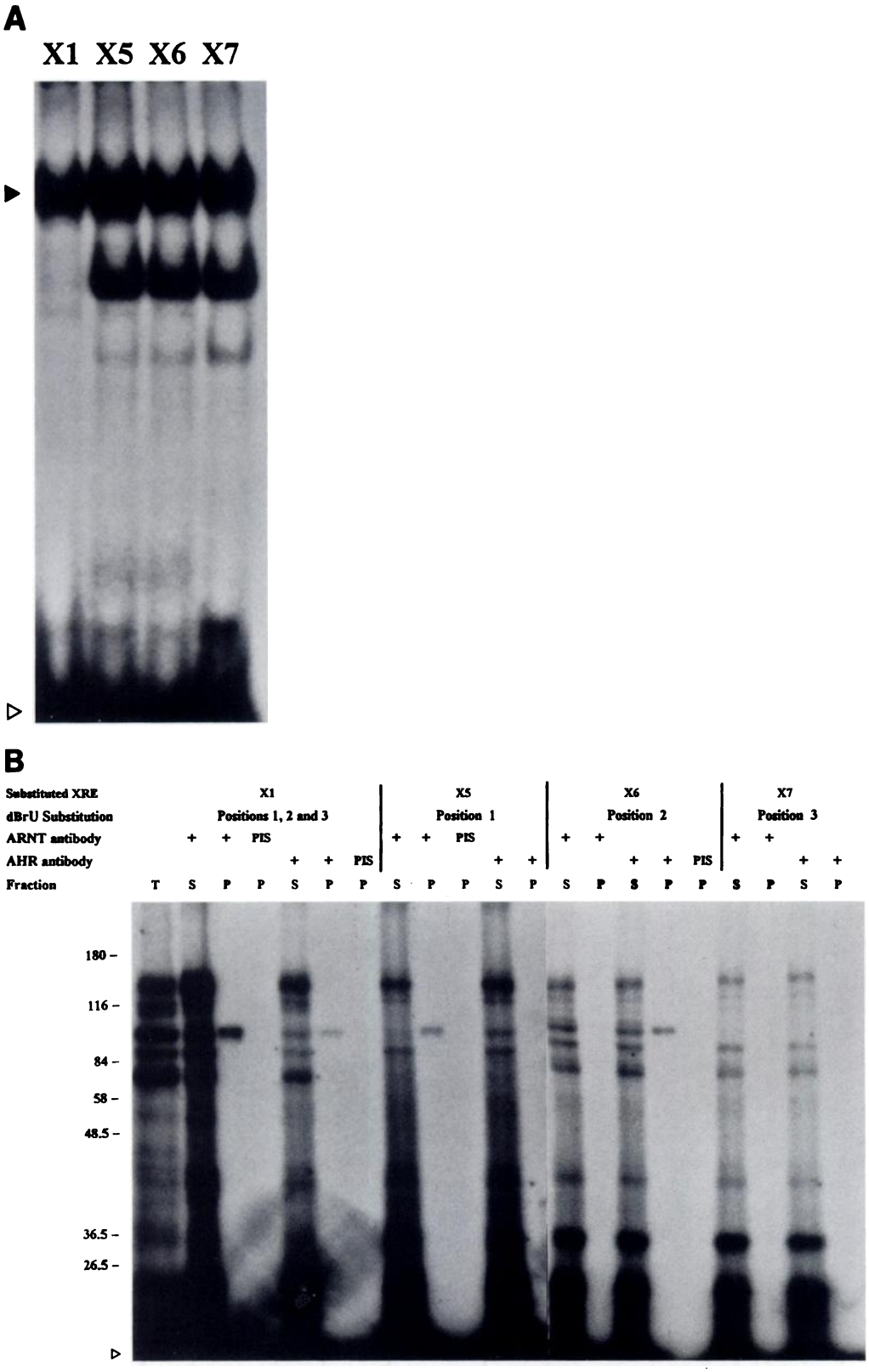

Fig. 2. EMSA and covalent cross-linking of the AHR complex using XREs with one dBrU substitution. A, An autoradiograph obtained after nondenaturing PAGE is shown. Control X1 and singly dBrU-substituted XREs (X5, X6, and X7) were tested with nuclear extracts from Hepa-1 cells grown with TCDD, by EMSA using 5 μg of protein and double-stranded ^{32}P-labeled XREs. B, X1 and singly dBrU-substituted XREs (X5, X6, and X7) were covalently cross-linked with a nuclear extract of TCDD-treated Hepa-1 cells and analyzed by immunoprecipitation as described in the legend to Fig. 1. Some samples were tested with IgG fractions of preimmune sera (*PIS*) corresponding to the AHR-specific and ARNT-specific antisera, as indicated. *T*, control covalent cross-linking reaction (without immunoprecipitation) boiled in SDS sample buffer and diluted before one third was resolved by SDS-PAGE. *Closed arrowhead*, position of the complex formed between the oligonucleotide and the transformed AHR complex; *open arrowheads*, position of free ^{32}P-labeled XRE. *Numbers to the left*, molecular mass standards (in kDa).

other bHLH proteins but the basic region of AHR conforms only poorly. In particular, ARNT, but not AHR, contains a glutamic acid residue that is conserved in other bHLH proteins and is known, from X-ray crystallographic analysis of certain of these proteins, to contact the CA residues of each E-box half-site (32–34).

The exact position in the XRE that demarcates the border between the AHR binding region and the ARNT binding region has not been determined. However, it is tempting to speculate that this lies between the cytosine residue and the guanine residue, as indicated (5′-C|GTG-3′), in the core of the XRE. If this is the case, then ARNT would bind to a complete

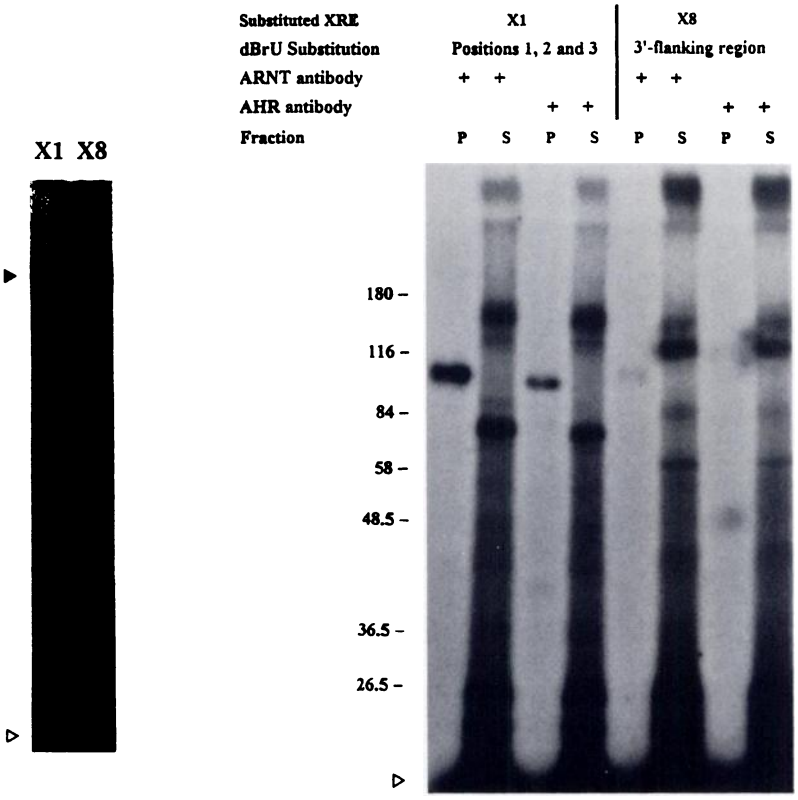

Fig. 3. EMSA and covalent cross-linking of the AHR complex using a XRE with flanking dBrU substitutions. *Left*, EMSA using nuclear extract (5 μg of protein) from Hepa-1 cells grown with TCDD and ^{32}P-labeled X1 or X8. *Right*, X1 and X8 covalently cross-linked with a nuclear extract of TCDD-treated Hepa-1 cells and analyzed by immunoprecipitation with antibodies to AHR and ARNT, as described in the legend to Fig. 1. *Closed arrowhead*, position of the complex formed between the oligonucleotide and the transformed AHR complex; *open arrowheads*, position of free ^{32}P-labeled XRE. *Numbers to the left*, molecular mass standards (in kDa).

E-box half-site and AHR would bind to the sequence 5′ to this. Neither subunit appears to loop over and contact nucleotides on the opposite side of its half-site, because all ARNT contacts occurred on nucleotides located 3′ to the aforementioned border in the XRE and the only AHR contact occurred on a nucleotide located 5′ to this border. Surprisingly, AHR bound at a position where the identity of the nucleotide does not have to be conserved for AHR complex binding to occur. It also did not appear to bind at any of the three positions immediately 5′ to this site, consistent with previous studies indicating that the identities of the nucleotides at these three positions make little, if any, difference with respect to the strength of AHR complex binding (15–17). We therefore suggest that AHR may play a less prominent role in DNA sequence identification than does ARNT. Structural analysis of several other bHLH-containing proteins has shown that helix 1 and the basic region form a continuous α-helix as the latter region contacts DNA (32–34, 36). The mouse, rat, and human AHRs contain an α-helix-interrupting proline residue in the middle of their nominal basic regions (37). The amino acids in the nominal basic region of AHR that are located immediately to the amino-terminal side of this proline residue may therefore not contribute to DNA binding.

Our results differ from those of Swanson *et al.* (38). Those authors observed that after *in vitro* transformation of guinea pig and rat liver cytosols, three proteins that were between 97 and 115 kDa in size could be cross-linked to a dBrU-substituted XRE sequence. One of the XRE-cross-linked proteins co-migrated, in nondenaturing electrophoresis, with AHR that had been cross-linked to an ^{125}I-labeled TCDD analog, and those authors proposed that this XRE-cross-linked protein was AHR. Using XRE sequences substituted with dBrU at various positions, Swanson and co-workers determined that the protein that they identified as AHR was cross-linked when the indicated thymine in the core of the XRE (5′-CGTG-3′) was substituted with dBrU. However, this is the position where we observed cross-linking of ARNT. Swanson and co-workers observed that the other proteins appeared to be cross-linked to the nucleotide where, in the present paper, we observed cross-linking of AHR. Because it is possible that AHR cross-linked to a XRE oligonucleotide migrates in nondenaturing gels differently from AHR cross-linked to ligand, a possible explanation for the differences between our findings and those of Swanson and co-workers is that those authors misidentified which XRE-cross-linked band corresponds to AHR. At least one of the other proteins identified by Swanson and co-workers is probably ARNT. The identity of the third protein is unknown. We did not obtain evidence for a third cross-linked protein in mice after AHR complex transformation *in vivo* or *in vitro* (7).

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