The Ah receptor (AHR), the Ah receptor nuclear translocator protein (ARNT), and single-minded protein (SIM) are members of the basic helix-loop-helix-PAS (bHLH-PAS) family of regulatory proteins. In this study, we examine the DNA half-site recognition and pairing rules for these proteins using oligonucleotide selection-amplification and coprecipitation protocols. Oligonucleotide selection-amplification revealed that a variety of bHLH-PAS protein combinations could interact, with each generating a unique DNA binding specificity. To validate the selection-amplification protocol, we demonstrated the preference of the AHR ARNT complex for the sequence commonly found in dioxin-responsive enhancers in vivo (TNGCGTG). We then demonstrated that the ARNT protein is capable of forming a homodimer with a binding preference for the palindromic E-box sequence, CACGTG. Further examination indicated that ARNT recognizes a relaxed partner specificity, since it was also capable of forming a heterodimer with SIM and recognizing the sequence GT(G/A)CGTG. Coprecipitation experiments using various PAS proteins and ARNT were consistent with the idea that the ARNT protein has a broad range of interactions among the bHLH-PAS proteins, while the other members appear more restricted in their interactions. Comparison of this in vitro data with sites known to be bound in vivo suggests that the high affinity half-site recognition sequences for the AHR, SIM, and ARNT are T(C/T)GC, GT(G/A)C (5-half-sites), and GTG (3-half-sites), respectively.

The AHR\(^1\) is a bHLH protein that mediates the metabolic, carcinogenic, and teratogenic effects of compounds such as TCDD (1). In response to agonists, the AHR interacts with a related protein known as ARNT to form a dimeric\(^2\) complex that is capable of binding genomic enhancer elements, known as DREs, and activating transcription at adjacent promoters (2–5). The AHR and ARNT have sequence similarities to two regulatory proteins found in Drosophila, SIM, and PER (6–10). SIM is a developmentally regulated bHLH protein involved in controlling central nervous system midline gene expression (11). PER lacks a bHLH domain and thus may be an inhibitor of the related signaling pathway involved in the maintenance of circadian rhythms (12). The hallmark of this family of proteins is that they all possess homology in a sequence of 200–300 amino acids termed a PAS domain (13). In the AHR, the PAS domain has been shown to be involved in ligand binding, interaction with Hsp90, and may serve as a secondary surface to support ARNT dimerization (2, 14–16).

Basic/helix-loop-helix proteins are involved in a variety of tightly regulated biological processes, such as the regulation of myogenesis (MyoD/E47) (17), neurogenesis (Achaete-scute/daughterless) (18), regulation of immunoglobulin genes (TFE3/TFE3) (19), cellular proliferation (Myc/Max) (20, 21), and xenobiotic metabolism (AHR/ARNT) (22). Biochemical and crystallographic data suggest that the HLH domains often act in concert with secondary dimerization surfaces (e.g. "leucine zippers" and possibly PAS domains) to position the two \(\alpha\) helical basic regions within opposing major grooves of B-DNA, generating a "scissor grip" structure with high affinity for the core DNA sequence, CANNTG (22–24). This DNA enhancer sequence is commonly referred to as an E-box and contains either CG or GC dinucleotides at the degenerate positions (i.e. CACGGT or CAGCGT) (25–28). Current models suggest that E-boxes can be viewed as containing two half-sites, with each partner’s basic region determining half-site specificity (e.g. the 5’-CAN or the NTG-3’ half-sites within 5’-CANNTG-3’). The multiplicity of half-sites and potential dimerization partners may allow production of a large number of homo- or heterodimeric pairs, each with unique sequence binding specificities and consequences for cellular signaling. In contrast to the recognition sites for most bHLH dimers, the cognate response element of the AHR-ARNT complex, the DRE, usually contains TNGCGT (5, 29–32). Unlike the E-box, the DRE is not palindromic, and thus the DNA half-site specificities of each protein are not readily apparent and are probably different.

In this study, we employed a DNA selection and amplification protocol to identify those bHLH-PAS protein combinations that could form productive DNA binding species and to characterize their individual DNA recognition sites. To validate the approach, we first demonstrated that the AHR-ARNT heterodimer would select the known DRE sequence from a pool of binding complexes of most bHLH proteins is dimeric (23, 24, 49). However, we cannot rule out the possibility that higher order complexes exist, such as dimers of dimers (tetramers), with each dimer independently interacting with separate DNA sites (22). The existence of such higher order complexes would not alter any of our conclusions.

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†To whom correspondence should be addressed: Dept. of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Ave, Chicago, IL, 60611. Tel.: 312-503-9855; Fax: 312-503-5349; E-mail: c-bradfield@nwu.edu.

\(^1\)The abbreviations used are: AHR, Ah receptor; bHLH, basic helix-loop-helix; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DRE, dioxin-responsive elements; PER, period; Hsp90, heat shock protein of 90 kDa; AHRCL316, AHR deletion in which 316 amino acid residues have been removed from the C terminus; AHRGN315, AHR construct in which the N-terminal 316 amino acids have been replaced by the DNA binding and dimerization domain of Gal4 (3); PCR, polymerase chain reaction.

\(^2\)In this manuscript, we refer to the DNA binding complexes of bHLH-PAS proteins as dimers. This assumption is based on x-ray crystallographic evidence demonstrating that the fundamental DNA
over 10^7 sequences. We then used this selection approach to demonstrate that ARNT also has the capacity to form homodimers as well as heterodimers with SIM, with each complex generating a unique DNA sequence binding specificity. Integration of the DNA selection and coprecipitation results allowed us to deduce the half-site specificities and pairing rules for the AHR, ARNT, and SIM.

**EXPERIMENTAL PROCEDURES**

**Materials**—The DNA expression plasmid, pSMMB40, was a gift from Dr. Stephen Crewe (University of North Carolina, Chapel Hill). The plasmids pRNA4H, pRNA4H-1C516, pRNA4H-1C515, and pRNA4H-1C514 were constructed as described previously (2, 33). The affinity-purified anti-ARNT polyclonal immunoglobulins were a gift from Dr. Alan Poland (34). The affinity-purified anti-AHR polyclonal immunoglobulins were a gift from Sima Nickels. The commonly recognized core DRE DNAsynthesizer (Foster City, CA). The western University Biotechnology Center using an Applied Biosystems 310 DNA sequencer (Foster City, CA).

Nickel-nitriloacetic acid resin was obtained from Qiagen (Chatsworth, CA). The commonly recognized core DRE DNAsynthesizer (Foster City, CA).

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The double-stranded oligonucleotide pool generated from OL187 was incubated with 10 fmol of reticulocyte lysate-expressed AHRΔ516 and ARNT. The mixture was subjected to the selection and amplification protocol, and the individual clones were sequenced. The most highly conserved sequence, GCCGTG, is boxed. B, tabulation of nucleotide frequencies at each position (n = 24). All frequencies were multiplied by 100. The frequencies of individual nucleotides were analyzed by χ-square at the p < 0.01 level. Frequencies above the expected random level are underlined. C, an AHR-ARNT consensus sequence derived from statistically significant nucleotides. D, a sample from the double-stranded oligonucleotide pool (OL187) was cloned and sequenced to verify equal representation of each nucleotide, and the frequencies were calculated and analyzed by χ-square (n = 19).

Statistical Analysis—χ-square goodness of fit test was used to determine whether frequencies of nucleotides at each position of the oligonucleotide were different than expected random frequencies (40). In the case where DNA selection and amplification-derived AHR-ARNT sequences were compared with those present in bona fide DREs, two by two contingency tables were used to compare frequencies of nucleotides. Significance for all tests was set at p < 0.01.

RESULTS

Validation of the DNA Selection and Amplification Strategy—To validate the DNA selection and amplification technique, we first examined the nucleotide specificity of the AHR-ARNT heterodimer. We amplified a pool of oligonucleotides, derived from OL187, to generate double-stranded oligomers that contained 13 consecutive random nucleotides, theoretically encoding greater than 7 x 10^7 unique sequences. The oligonucleotides that specifically bound to the AHR-ARNT complex were subjected to three rounds of selection and amplification. 24 selected oligonucleotides were cloned and sequenced (Fig. 1A). Statistical analysis by χ-square was performed to identify those nucleotides preferentially selected for by the AHR-ARNT complex (Fig. 1B). Nucleotides that occurred at greater than expected frequencies (p < 0.01) were used to derive a consensus recognition sequence, TNGCGTGC (Fig. 1C). Of these oligonucleotides, 22 contained the GCCGTG core sequence that is commonly found in bona fide DREs. Two oligonucleotides, AHA23 and AHA24, contained similar core motifs, TCGTG and GTGTCG, respectively. Subsequent gel shift analysis indicated that these two sequences were capable of binding AHR-ARNT complexes, albeit at lower affinities than those sites containing the complete core motif, GCCGTG (results not shown). Analysis of the OL187 oligonucleotide pool that was cloned, amplified, and sequenced directly, without selection by the AHR-ARNT heterodimer, served as a control. χ-square analysis of these sequences indicated that the AHR-ARNT selected sequence was not the result of biased oligonucleotide synthesis (Fig. 1D).

Analysis of Sequences Flanking the GCCGTG Motif—As shown in Fig. 1, the position of the GCCGTG was biased toward the 3'-end of the oligonucleotide. To determine if this bias was the result of flanking nucleotides, we constructed an additional nucleotide pool (OL224) that fixed the core motif, GCCGTG, between seven random nucleotides on the 3'- and 5'-ends. This oligonucleotide pool, containing approximately 3 x 10^8 possible sequences, was subjected to three rounds of the selection and amplification protocol, and the selected oligonucleotides were sequenced (Fig. 2A). χ-square analysis was performed (Fig. 2B) and indicated that nucleotide preference occurred at 11 of the 14 flanking positions, resulting in a consensus sequence of GNNATGTGGCTGC (underlined sequences are fixed, Fig. 2C). Again, analysis of the control oligonucleotide pool indicated that nucleotide preference was not the result of biased oligonucleotide synthesis (Fig. 2D). To confirm that our consensus sequence was highly specific for the AHR-ARNT complex, we synthesized the corresponding oligonucleotide (OL318/319) and performed gel shift analysis. As demonstrated in Fig. 3, complex formation required both proteins. Neither ARNT nor AHRΔ516 recognized this motif alone (Fig. 3, lanes 1-3), recognition of the consensus sequence by full-length AHR and ARNT was ligand responsive (Fig. 3, lanes 4 and 5), and the complex was recognized by anti-ARNT and anti-AHR immunoglobulins (Fig. 3, lanes 6 and 7). Addition of purified immunoglobulin did not affect the migration of the AHR-ARNT complex (Fig. 3, lane 8).

Comparison of the AHR-ARNT Selected Sequence With Bona Fide Enhancer Elements—To support the idea that our strategy would select for biologically relevant DNA binding motifs, we compared the consensus sequence selected by the
AHR:ARNT complex in vitro to sequences known to correspond to functional enhancers in vivo. For this comparison, we first analyzed 10 bona fide DREs to determine the frequency of nucleotides at each position (Fig. 4). These frequencies were then compared to the corresponding frequencies observed in the selected and amplified oligonucleotides (Fig. 4D). The in vitro derived consensus was similar to the bona fide DREs at 14 out of 19 of the nucleotide positions. Statistically significant differences were detected at the outer most positions (−8, −9, 9, 10) and at the −5 position.

Selection and Amplification of ARNT-Homodimer Recognition Sequences—Purified ARNT obtained from baculovirus-infected SF9 cells (36) with the addition of unprogrammed reticulocyte lysate was subjected to the same DNA selection and amplification protocol described above, using double-stranded oligonucleotides generated from OL187. After four rounds of selection and amplification, 20 ARNT-specific sequences were aligned and analyzed by $\chi^2$-square to yield a consensus sequence, CACGTG (Fig. 5). Unlike the oligonucleotides selected from OL187 by the AHR-ARNT complex, no bias was observed due to flanking nucleotides, and no statistically significant specificities were observed for nucleotides that flanked this core (Fig. 5B). Four sequences that contained the AAGC bend (AA17, AA18, AA19, AA20) motif were also amplified. Gel shift analysis demonstrated that these sequences were recognized by the ARNT complex but at a lower affinity than sequences containing the CACGTG sequence (data not shown). To confirm that the derived consensus sequence, CACGTG, was specific for ARNT homodimers, we synthesized the corresponding consensus oligonucleotide, and demonstrated that a specific ARNT-DNA complex was formed in gel shift analysis (Fig. 6A, lane 2). The presence of ARNT in the complex was confirmed by supershifting the complex in the presence of anti-ARNT immunoglobulin (Fig. 6A, lane 3). Finally, we confirmed that the ARNT-DNA binding complex could be formed at the lower concentrations of ARNT that are typically generated in the reticulocyte lysate expression system and that may also be found in cells (i.e., −1 fmol/μl) (Fig. 6B, lane 1).

ARNT and SIM Interact Resulting in Unique DNA Binding Specificity—The selection and amplification protocol was performed to determine if ARNT could interact with SIM and recognize a specific DNA sequence. Using the oligonucleotide pool derived from OL187, we were unable to select and amplify a discrete SIM-ARNT-DNA complex that was dependent on the presence of both proteins. We repeated the procedure using OL224 as the oligonucleotide source (see "Discussion"). Following four rounds of selection and amplification, a pool of specific SIM/ARNT selected DNA was cloned and sequenced. Given the apparently weak interaction of the complex and the comigration of non-specific protein-oligonucleotide species, 80 of the selected sequences were radiolabeled, and each was individually reanalyzed by gel shift analysis to confirm its interaction with both SIM and ARNT. Of the 80 amplified oligonucleotides,
19 were specific for the SIM-ARNT complex as judged by the formation of specific gel shift bands that were detected only in the presence of both proteins and that were recognized by the ARNT-specific antibodies (Fig. 7A). Nucleotides that were associated with the SIM complex formation were identified by x-square analysis and were used to derive a consensus sequence, GNNNNGTGCGTGANNNTCC (Fig. 7, B and C). Gel shift analysis using an oligonucleotide corresponding to the derived consensus sequence (OL331/332) confirmed that it was specific for the SIM-ARNT complex (Fig. 8A). Again, complex formation required both proteins, since neither ARNT nor SIM could recognize the sequence alone (Fig. 8A, lanes 1–3), and the complex was recognized by ARNT-specific antibodies but not purified IgG (Fig. 8A, lanes 4 and 5).

While this work was in review, a report described a consensus sequence found upstream of SIM-regulated genes in...
Drosophila, GTACGTG (41). This core sequence differed by a single nucleotide from the sequence deduced by our in vitro approach (i.e. GTACGTG versus GTGCAGT). Since our selected SIM-ARNT sequence was biased for a G at this position due to the use of oligonucleotides with a fixed GC repeat core, we chose to examine the impact of this single nucleotide difference on binding by the SIM-ARNT complex. To control for effects of adjacent sequences, we engineered oligonucleotides that contained these two core sequences into flanking sequences derived from either the SIM or AHR DNA recognition sites. To increase the sensitivity of these attempts, experiments were also performed using baculovirus-expressed AHR. All combinations were repeated three times without detection of a specific DNA binding complex. In addition, we synthesized oligonucleotides containing a palindrome of the predicted recognition half-sites of the AHR and SIM (core sequences of T(C/T)GC and GTGCGTGA, respectively). Gel shift analysis indicated that either the AHR or SIM with these radiolabeled oligonucleotides failed to yield specific DNA binding complex formation (data not shown).

DNA Binding Specificity of bHLH-PAS Dimers for Their Selected Consensus Sequences and Various E-boxes—As an additional demonstration of DNA binding specificity, we used competitive binding analysis to compare the affinities of bHLH-PAS dimers for oligonucleotides corresponding to their consensus DNA sequences and a variety of E-boxes. Competitive binding analysis with each productive bHLH-PAS pair (i.e. AHR-ARNT, ARNT-ARNT, or SIM-ARNT) demonstrated that each DNA binding complex had the greatest affinity for its derived consensus sequence over all of the E-box sequences tested (see Fig. 9, A–C). Presence of the ARNT homodimer consensus sequence (OL329/330) diminished the complex formation in all reactions that contained the ARNT protein (Fig. 9, A and C, lane 3). The ARNT homomeric species demonstrated the greatest affinity for the E-box CACGTG (Fig. 9B, lanes 3 and 5), with much lower affinity for the TNGCGTG being the 5’-half-site of the AHR and GT(G/A)C being the 5’-half-site of SIM.

FIG. 7. Determination of SIM-ARNT DNA recognition sites. A, Double-stranded OL224 containing the fixed sequence, GGCGTG, and flanked by seven random nucleotides was incubated with 1 fmol each of reticulocyte lysate-expressed SIM and ARNT, the mixture was subjected to four rounds of DNA selection and amplification, and the individual clones were sequenced. The most highly conserved sequence, GTTGCGTA, is boxed. B, Tabulation of the nucleotide frequencies at each position (n = 19). All frequencies were multiplied by 100. The frequencies of individual nucleotides were analyzed by χ²-test at the p < 0.01 level. Frequencies above the expected level are underlined. C, A SIM-ARNT consensus sequence derived from statistically significant nucleotides.

TABLE 3. DNA Binding Specificity of bHLH-PAS Dimers for Their Selected Consensus Sequences and Various E-boxes.

| E-boxes | AHR | ARNT | ARNT | SIM |
|---------|-----|------|------|-----|
| TGGAGTG | 9   | 9    | 9    | 9   |
| TGGAGTG | 9   | 9    | 9    | 9   |
| TGGAGTG | 9   | 9    | 9    | 9   |
| TGGAGTG | 9   | 9    | 9    | 9   |
sequence (Fig. 9B, lane 2) or the other E-boxes, CAGCTG or CATGTG (Fig. 9B, lanes 6 and 7).

Relative DNA Binding Affinities of AHR-, ARNT-, and SIM-containing Complexes—To obtain estimates of the relative DNA binding affinities of the full-length AHR-ARNT, ARNT-ARNT, and SIM-ARNT complexes, we performed dissociation rate analysis using the gel shift assay as an end point. As shown in Fig. 10, the calculated half-life values of the full-length AHR-ARNT and ARNT-ARNT complexes are similar (3.2 versus 5.06 min) while that of the SIM-ARNT complex was considerably more rapid (less than 0.2 min).

Demonstration of PAS Protein Interactions by Coprecipitation—To further establish the interaction of bHLH-PAS proteins, we utilized a coprecipitation assay (Fig. 11). Protein-protein interactions of ARNT-AHR, ARNT-AHRΔ516, and ARNT-ARNT, but not ARNT-SIM, were observed. Specificity of the ARNT-containing interactions was demonstrated by the lack of coprecipitation using the 35S-labeled GN1315 AHR construct in which most of the dimerization domain has been replaced by the DNA binding and dimerization domain of Gal4 (2). Interestingly, ARNT-ARNT interactions were observed only when the incubations contained the CACGTG-containing oligonucleotides.

**DISCUSSION**

Strategy—Our hypothesis was that bHLH-PAS proteins could form a variety of heteromeric and homomeric combinations and that each complex would display unique oligonucleotide binding specificities. We predicted that the analysis of these different recognition sites would allow us to deduce the half-site specificity of each protein. To test these ideas, we utilized a DNA selection and amplification strategy to identify the preferred recognition sequences of various AHR, ARNT, and SIM combinations (27). The oligonucleotides bound by these protein complexes were isolated from pools of millions of independent, unbound sequences. Once selected by the protein complex, the oligonucleotides were isolated from nondenaturing polyacrylamide gels and amplified by PCR. To increase the specificity of the method, the oligonucleotide pools were typically subjected to multiple rounds of selection and amplification prior to cloning and sequence analysis. The power of this method arises from the fact that it is independent of any prior knowledge or preconceptions regarding DNA binding specificity and has the potential to yield information about protein-DNA interactions not readily attainable by more conventional methods such as DNA footprinting or site-directed mutagenesis of a single oligonucleotide sequence.

Specific versus Nonspecific Interactions—A number of approaches were used to ensure that amplified sequences were specific for the protein complex and not simply sequences that were nonspecifically comigrating in the gel. First, bands of amplified oligonucleotides were analyzed (considered specific) only if the band was dependent upon the presence of all of the bHLH-PAS proteins used in the assay. Second, specificity was confirmed by the capacity of ARNT- or AHR-specific antibodies to supershift the radiolabeled complex. Third, a consensus was deduced from each set of selected oligonucleotides, and this information was used to design consensus oligonucleotides that were used in gel shift assays to confirm specificity of interaction. Only in the case of SIM-ARNT sequences was the presence of a comigrating nonspecific oligonucleotide observed. In this case, we reanalyzed each of the 80 amplified oligonucleotides independently by gel shift analysis to eliminate any nonspecific sequences (see above).

Validation of the DNA Selection and Amplification Strategy—To validate our strategy, we first employed this technique using the AHR-ARNT complex that recognizes the DRE sequence, TNGCGTG (5, 29–32). We anticipated one of two outcomes. Either the AHR-ARNT complex would recognize sequences containing this known core and validate our hypothesis that the other bHLH proteins used in the assay. Second, specificity was confirmed by the capacity of ARNT- or AHR-specific antibodies to supershift the radiolabeled complex. Third, a consensus was deduced from each set of selected oligonucleotides, and this information was used to design consensus oligonucleotides that were used in gel shift assays to confirm specificity of interaction. Only in the case of SIM-ARNT sequences was the presence of a comigrating nonspecific oligonucleotide observed. In this case, we reanalyzed each of the 80 amplified oligonucleotides independently by gel shift analysis to eliminate any nonspecific sequences (see above).

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**Fig. 8.** Gel shift analysis of the SIM-ARNT DNA binding complex. Approximately 0.5 fmol of reticulocyte lysate-expressed SIM and 4 fmol of baculovirus-expressed ARNT were subjected to gel shift analysis using OL333/332, which contained the derived SIM-ARNT consensus sequence (lane 2). A, specificity of SIM-ARNT heterodimer recognition of the derived consensus sequence. The incubation mixtures contained ARNT with 5 μl of unprogrammed reticulocyte lysate (lane 1), SIM alone (lane 2), ARNT and SIM (lane 3), ARNT and SIM with the anti-ARNT immunoglobulin (lane 4), and ARNT and SIM with purified IgG (lane 5). The arrow indicates the SIM-ARNT DNA binding complex. B, gel shift analysis of SIM and ARNT using 32P-labeled oligonucleotides corresponding to different flanking and core sequences. Gel shift experiments were performed with SIM alone (lanes 2, 5, and 8), ARNT alone (lanes 3, 6, 9, and 12), or both SIM and ARNT (lanes 1, 4, 7, 10) using 32P-labeled OL333/332 (GGGATGCGTGACATTC, lanes 1–3), OL464/465 (GGGATGCGTGACATTC, lanes 4–6), OL501/502 (AATTGTACGTGCCACAGA, lanes 7–9), or OL503/504 (AATTGTACGTGACAGA, lanes 10–12). Unprogrammed reticulocyte lysate was added, if necessary to normalize the amount of lysate in each reaction.
frequencies at various positions revealed a consensus sequence of TNGCGTGC. This sequence was essentially identical to the previously described DRE, TNGCGTG (5, 29–32). No sequences conforming to E-boxes were found in any of the 24 clones that were sequenced.

The analysis presented in Fig. 1 indicated that the positioning of the TNGCGTG core sequence within the random 13-mer was biased by the flanking sequences required for annealing PCR primers (i.e., most core sequences were found closer to the 3'-end of the oligonucleotide, Fig. 1A). This observation led us to examine the impact of flanking sequences on AHR/ARNT DNA binding specificity. The analysis using OL224 as the oligonucleotide pool revealed a consensus binding sequence of TNGCGTGC.

Fig. 9. Specificity of DNA recognition by AHR-ARNT, ARNT-ARNT, and SIM-ARNT complexes. Gel shift analysis of incubation mixtures containing reticulocyte lysate-expressed ARNT and AHR (0.5 fmol of each protein) with OL318/319 as the probe (A), baculovirus-expressed ARNT (4 fmol) and 10 μg of unprogrammed reticulocyte lysate with OL329/330 as the probe (B), or reticulocyte lysate-expressed SIM and ARNT (0.5 fmol of each protein) with OL331/332 as the probe (C) and 100-fold molar excess of the indicated competitor oligonucleotides: lane 1, none; lane 2, OL318/319; lane 3, OL329/330; lane 4, OL331/332; lane 5, OL321/322; lane 6, OL323/324; and lane 7, OL316/317.

Fig. 10. Dissociation rate analysis of the full-length AHR/ARNT, ARNT/ARNT, and SIM/ARNT DNA binding complexes. Each binding reaction containing the indicated proteins was allowed to come to binding equilibrium with 1 ng of the appropriate radiolabeled oligonucleotide (i.e., the derived consensus sequence of each DNA binding complex). Excess of unlabeled oligonucleotide was added to the mixture, and aliquots were removed at the indicated time points. Each value represents the average of two independent experiments ± S.E. See “Experimental Procedures” for details.

Fig. 11. Coprecipitation analysis of AHR-ARNT, ARNT-ARNT, and SIM-ARNT interactions. [35S]Labeled full-length AHR (top left), AHRΔ516 (top middle), AHRGN315 (top right), ARNT (bottom left), and SIM (bottom right) were coprecipitated in the presence (+) or absence (−) of the baculovirus-expressed six histidine-tagged ARNT (ARNT-his) using nickel-nitriloacetic acid resin in the presence or absence of the following: 10 μM β-naphthoflavone (top left), OL318/319 (top middle), OL329/330, OL316/317, OL323/324 (bottom left), and OL331/332 (bottom right).
GGNNAT(C/T)GCGTGACANNCC (Fig. 2).4 Nucleotides that were present at frequencies above expected random values were identified at 11 of the 14 flanking positions, including those in positions −4, −3, 4, 5, and 6. These results are consistent with those obtained using substitution mutagenesis of a DRE-containing oligonucleotide (31, 32). The selection of flanking nucleotides suggests that both the AHR and ARNT (or other proteins within this complex) are capable of DNA contacts at sites adjacent to the commonly recognized core sequence. In addition, our results suggest that positions not identified previously, the −9, −8, −7, −5, 9, and 10 positions, are selected for and thus could also play a role in the AHR-ARNT-DNA recognition.

If binding affinity is the only determinant of a functional DRE in vivo, then our consensus sequence for the AHR-ARNT complex should be identical to bona fide DREs. In an attempt to address this question, we compared our selected sequences to 10 DREs known to function upstream of TCDD-regulated genes. Since similarity is a difficult assertion to prove statistically, we identified those nucleotides that were statistically different. The most interesting discrepancy between the in vitro and in vivo consensus is the preference for an A at position −5 for the in vitro derived sequence and the lack of an A at −5 in any reported DRE. The absence of A at −5 may be an indication that inappropriate contacts are occurring in vitro, that additional proteins are required for in vivo interactions, or that some attenuation of binding affinity is required for optimal control of gene expression in vivo.

DNA Recognition by ARNT Homodimers—In an effort to determine half-site recognition of ARNT and to determine if ARNT could recognize a specific DNA sequence as a homodimer or as a heterodimer with other bHLH-PAS partners, we performed a series of selection and amplification experiments with various combinations of the AHR, ARNT, and SIM. The observation that ARNT is not found in association with Hsp90 (42) suggests that ARNT homodimeric complexes could specifically address this question, we compared our selected sequences to 10 DREs known to function upstream of TCDD-regulated genes. Since similarity is a difficult assertion to prove statistically, we identified those nucleotides that were statistically different. The most interesting discrepancy between the in vitro and in vivo consensus is the preference for an A at position −5 for the in vitro derived sequence and the lack of an A at −5 in any reported DRE. The absence of A at −5 may be an indication that inappropriate contacts are occurring in vitro, that additional proteins are required for in vivo interactions, or that some attenuation of binding affinity is required for optimal control of gene expression in vivo.

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rapid dissociation rate of the SIM-ARNT-DNA complex (Fig. 10) indicate that the SIM-ARNT interaction is relatively weak. The weak interaction of the SIM-ARNT complex found in this study is in contrast to that reported by Sogawa et al. (43).

Recently, a number of SIM-responsive elements have been cloned from Drosophila using an enhancer trapping technique (41). Sequence alignment of these regulatory elements revealed a consensus motif, (G/A)(T/A)ACGTG. This sequence differs by (41). Sequence alignment of these regulatory elements revealed a consensus motif, (G/A)(T/A)ACGTG. This sequence differs by 2 nucleotides when compared to the SIM-ARNT consensus core sequence we describe in Fig. 7, GTGCCTG. The difference exists at the −2 position (underlined) within the putative SIM binding 5′-half-site (A versus G). To examine the importance of this nucleotide position, we performed a series of gel shift experiments to determine the impact that this nucleotide had on SIM-ARNT recognition. We found that both A and G at the −2 position are specifically bound by the SIM-ARNT complex (Fig. 8B). Our inability to predict an A nucleotide at this position arose from our use of OL224 that has a fixed GCGTG core (see above). Thus, we conclude that the in vitro SIM-ARNT consensus core sequence is more appropriately GT(A/G)CGTG, with GTACGTG possibly having greater relevance to SIM-responsive gene regulation in vivo.

Half-site Recognition of ARNT, AHR, and SIM—The identification of half-site recognition of ARNT, AHR, and SIM in combination with analysis of the amino acid sequences of their basic regions should provide insights into the relationships between the bHLH-PAS proteins and members of other bHLH families. Interestingly, the ARNT-specific sequence half-site is also recognized by other bHLH proteins such as Max (44), Myc (45), and USF (22, 46, 47). The bHLH proteins that bind the 3′-half-site GTG sequence (binding CACGTG as homodimers) have been denoted as class B proteins and are distinguished by the presence of an arginine (R) residue in their basic region immediately following the sequence ERRR (i.e. ERRRR) (48) (Fig. 12). The bHLH proteins that lack this C-terminal Arg residue commonly recognize the 3′-half-site CTG sequence (binding CAGCTG) and are denoted class A. Our results suggest that ARNT is a class B protein since its homomorphic form recognizes the palindromic CACGTG sequence with greatest affinity, and its basic region has an Arg residue at the characteristic position. In addition, many bHLH proteins possess a critical glutamic acid residue (ERRR), which has been shown to contact the CA of the E-box sequence CANNTG (49). Although this residue is present in the basic region of ARNT, it does not occur at corresponding positions in either the AHR or SIM proteins. Thus, by predictions derived from these rules and from their primary amino acid sequences, neither the AHR nor SIM proteins would be expected to bind any known E-box half-sites. Our results support this prediction and suggest that when complexed with ARNT, the AHR has the greatest affinity for the 5′-half-site T(C/T)GC, and SIM has the greatest affinity for the half-site GT(A/G). We suggest that these proteins represent a unique class of bHLH proteins and designate this group as class C. While this paper was in review, another group determined the position of ARNT as the 3′-GTG half-site of the DRE (50).

Pairing Rules of bHLH-PAS DNA Binding Complexes—Our results indicate that certain rules dictate pairing and subsequent DNA binding of bHLH-PAS proteins. In contrast to the identification of DNA binding complexes formed with ARNT alone, AHR and ARNT, or SIM and ARNT, no oligonucleotide sequences could be selectively amplified when the AHR and SIM (each alone or mixed) were used as the binding species. These experiments were repeated multiple times, using either OL187 or OL224 and the higher concentrations of protein that were attainable with baculovirus-expressed AHR. The fact that heterodimeric binding of the bHLH-PAS proteins was detected only with ARNT suggests that ARNT may be a general dimerization partner for PAS proteins that respond to cellular signals. In addition, the multiplicity of productive bHLH-PAS protein combinations may have a significant impact on the spectrum of DNA binding sites, enhancer elements, and responsive genes affected by these proteins in the presence and absence of compounds such as TCDD. A second explanation for the limited number of bHLH-PAS protein pairs that were detected by this method cannot be ruled out. Our inability to detect AHR or SIM homodimeric or AHR-SIM heterodimeric interactions with DNA may be due to a failure of the method to detect weaker protein-protein or protein-DNA interactions in vitro.

Summary—These data support several important conclusions. First, ARNT is capable of forming distinct DNA binding complexes with another molecule of ARNT, the AHR, or SIM. This suggests that bHLH-PAS proteins may be involved in a combinatorial mechanism of gene regulation that involves the formation of multiple homo- or heterodimeric pairs, each with a role in controlling expression of distinct batteries of genes (51). For example, the observation that ARNT may interact with E-box elements suggests that in the absence of AHR agonists, ARNT homodimers play a role in the regulation of a second battery of genes, possibly through interactions at E-boxes that may be down-regulated in the presence of TCDD. Second, since ARNT is capable of recognizing DNA as a component of several distinct complexes, we were able to elucidate the DNA recognition half-sites of these PAS proteins. As predicted by amino acid sequence homology to other class B bHLH proteins, ARNT recognizes the 3′-half-site GTG. In contrast, the basic region amino acid sequences of both the AHR and SIM are unique and specify distinct 5′-half-sites, T(C/T)GC and GT(A/G)GC, respectively. Finally, the AHR-ARNT complex displays a preference for nucleotides that flank the core T(C/T)GC motif, suggesting that the protein-DNA interactions of this complex extend beyond the core motif. Other PAS protein complexes (i.e. ARNT-ARNT or SIM-ARNT) display fewer preferences for flanking nucleotides, suggesting that the sequence specificity of various PAS protein complexes may differ substantially or may be less restricted than that of the AHR-ARNT complex.

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