0026-895X/98/040671-07$3.00/0

MOLECULAR PHARMACOLOGY, **54:**671–677 (1998).

The Aryl Hydrocarbon Receptor Interacts with Transcription Factor IIB

HOLLIE I. SWANSON and JUN-HAU YANG

Department of Pharmacology, University of Kentucky, Lexington, Kentucky 40536

Received March 20, 1998; Accepted July 2, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

The aryl hydrocarbon receptor (AHR) and its DNA binding partner, the AHR nuclear translocator (ARNT), are basic helix-loop-helix transcription factors that mediate many of the toxic and carcinogenic effects of polyhalogenated aromatic hydrocarbons. The basic regions of the AHR and ARNT contact the GCGTG recognition site, whereas both their helix-loop-helix domains and periodicity-ARNT-single-minded domains participate in heterodimerization. To delineate the transcription factors that may facilitate DNA binding and transcriptional activation of the AHR/ARNT heterodimer, we questioned whether transcription factor IIB (TFIIB) may interact with either the AHR or ARNT and whether this interaction may affect the ability of the AHR/ARNT complex to bind DNA. Coaffinity precipitation assays demonstrated that both the AHR and ARNT were capable of interacting with TFIIB. Domain mapping experiments revealed that TFIIB interacts with the periodicity-ARNT-single-minded and carboxyl-terminal regions of the AHR. To determine whether the interaction between TFIIB and the AHR may affect DNA binding of the AHR and ARNT complex, we performed gel shift experiments in the absence and presence of TFIIB. The addition of TFIIB significantly increased the formation of the AHR/ARNT DNA binding complex, but only if TFIIB was first allowed to interact with the AHR before the addition of ARNT. These results indicate that TFIIB interacts with the AHR and may stabilize the DNA binding form of the AHR and thereby augment the ability of the AHR/ARNT complex to interact with its DNA recognition site.

The AHR and its DNA binding protein ARNT are bHLH proteins that are distinguished by a secondary dimerization region termed the PAS domain (Jackson *et al.*, 1986; Hoffman *et al.*, 1991; Nambu *et al.*, 1991; Burbach *et al.*, 1992). The bHLH/PAS proteins are a family of transcription factors that are involved in a number of diverse functions. For example, the *Drosophila melanogaster* single-minded protein, SIM, regulates formation of the central nervous system (Muralidhar *et al.*, 1993). Interestingly, the locus of the mammalian homologue, SIM2, coincides with the Down syndrome chromosomal region (Chen *et al.*, 1995). Both the *D. melanogaster* (Konopka *et al.*, 1971) and mammalian PER (Tei *et al.*, 1997), as well as the mammalian clock (Vitaterna *et al.*, 1994) proteins regulate circadian rhythms. The hypoxia inducible factor 1α and its heterodimerization partner, hypoxia inducible factor 1β (ARNT) mediate the cellular response to reduced oxygen levels (Wang *et al.*, 1995). In addition, several bHLH/PAS proteins function as coactivators and mediate agonist-induced transcriptional activation. For example, Src1 (Onate *et al.*, 1995), GRIP1/TIF-2 (Voegel *et al.*, 1996; Hong *et al.*, 1997) and RAC3 (Li *et al.*, 1997) facilitate the transcriptional activation of a number of steroid hormone receptors. Finally, the AHR (Burbach *et al.*, 1992) and its heterodimerization partner, ARNT (Hoffman *et al.*, 1991), regulate xenobiotic metabolizing genes. Additional bHLH/PAS family members with as yet uncharacterized functions may shed new insights into the physiological roles of this class of transcription factors (Hogenesch *et al.*, 1997).

Like many bHLH proteins, the AHR and ARNT proteins contact DNA via their basic regions that are located at their amino-termini and lie adjacent to the HLH/PAS regions (Dolwick *et al.*, 1993; Reisz-Porszasz *et al.*, 1994). Although the transcriptional activity of the AHR and ARNT have been mapped to glutamine-rich regions that lie within their carboxyl-termini, the biochemical events that mediate the transcriptional activities of these two proteins are poorly understood (Jain *et al.*, 1994; Ma *et al.*, 1995).

The AHR interacts with high specificity and affinity to the prototypical ligand, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (For

This work was supported by the University of Kentucky Research Fund (Grant 847) and National Institutes of Health Grant ES08088. This work was presented at the Society of Toxicology annual meeting in March 1998.

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; SIM, single minded; PER, period; PAS, PER-ARNT-SIM homology region; bHLH, basic helix-loop-helix; Src1, steroid receptor coactivator-1; GRIP1, glucocorticoid receptor interacting protein 1; TIF-2, transcriptional mediators/intermediary factor 2; RAC3, receptor-associated coactivator 3; Hsp90, 90-kDa heat shock protein; DRE, dioxin responsive element; TFIIB, transcription factor IIB; TFIID, transcription factor IID; VP16, herpes virus protein 16; oct1, octamer binding protein 1; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; GST, glutathione S-transferase.

review, see Swanson and Bradfield, 1993). In its unactivated form, the AHR exists in the cytosol complexed to a number of proteins, including a dimer of Hsp90. However, after agonist binding, the AHR translocates to the nucleus, where it dimerizes with ARNT. This heterodimeric pair interacts with specific DNA sequences termed DREs, resulting in the activation of a number of genes, including *CYP1A1*. The increased transcriptional levels of targeted promoters that are mediated by activator proteins, such as the AHR and ARNT, may be a result of several possible actions (Goodrich *et al.*, 1996). The activator protein(s) may 1) recruit the general transcription factors to the promoter, 2) exert a conformational change upon the nucleoprotein complex at the promoter, or 3) stimulate a modification such as a phosphorylation event on the promoter-bound proteins. Each of these actions may be facilitated by protein-protein interactions between the activator protein and a transcriptional coactivator or a general transcription factor, such as TFIIB or TFIID.

The idea that TFIIB plays a critical role in transcriptional activation is illustrated by the fact that TFIIB interacts with the activator protein VP16, thereby increasing the rate limiting step of transcription, assembly of TFIIB into the preinitiation complex (Lin and Green, 1991). Further, mutations within the transcriptional activation domain of VP16 that disrupt the TFIIB-VP16 interaction yield variant VP16 proteins that are incapable of transcriptional activation (Lin and Green, 1991). A diverse group of activator proteins that have been demonstrated to directly contact TFIIB include the nuclear hormone receptors (vitamin D, thyroid, estrogen, and progesterone receptors), nuclear factor κB65, VP16, and Oct 1 (Lin and Green, 1991; Ing, *et al.*, 1992; Baniahmad *et al.*, 1993; Blanco *et al.*, 1995; Nakshatri *et al.*, 1995; Schmitz *et al.*, 1995). In an effort to understand the mechanisms by which the AHR and ARNT may activate their target genes, we questioned whether either the AHR or ARNT may directly contact TFIIB. In this study, we demonstrate that both the AHR and ARNT interact directly with TFIIB. In addition, we have determined that the interaction between TFIIB and the AHR includes two contact sites: one that occurs within the PAS domain and a second that occurs within the carboxyl terminus of the AHR. Finally, we demonstrate that the interaction of TFIIB with the AHR does not interfere with its ability to interact with ARNT or DNA; rather, it increases DNA binding of the AHR/ARNT complex.

Experimental Procedures

Oligonucleotides. Oligonucleotides were purchased from Gibco BRL (Gaithersburg, MD). The annealed oligonucleotides that were used as the radiolabeled probe for the gel shift assay and contain the DRE (underlined) are: 5′- TCGAGCTGGGGGCATTGCGTGACATAC (HIS 17) and 3′-TCGAGGTATGTCACGCAATGCCCCCAGC (HIS 18). This sequence has been determined previously to be the optimal DNA recognition site of the AHR and ARNT complex (Swanson *et al.*, 1995). The following oligonucleotides were used as polymerase chain reaction primers:
HIS 34, GCGACTAGTCACCATGTTCTTTGATGTTGCATTAAAGTCC; HIS 39, GCACTAGTCTAAGCTTCCTGAAAGATG; HIS 40, GCACTAGTCCTCTACAAATGTGGTATGGC; HIS 50, CCCAAGCTTATAGCTGTGGTAGTTTGTCCACTG; HIS 51, CGCACTAGTACCATGGCTAGCACCAGTCGTTTG; HIS 61, GCGGATCCATGAGCAGCGGCGCCAAC; HIS 62, GCGGATCCCAGCCGGTCTCTGTGTCGCTT; HIS 68, GCACTAGTACCATGGGAGTGGACGAGCCCATG; HIS 88, GCACTAGTACCATGTTCACACCTATTGGTTGTGATGC; HIS 123, CCCAAGCTTACGCGTGGAAGTCTAGCTTGTGTTTGG; HIS 130, CCAAGCTTATTGCTGGGGGCACACCATC.

Materials. The plasmids pmuAHR, pmuAHRCΔ516 (denoted as $AHR_{(1–289)}$ in the present study), and phuARNT were constructed as described previously (Dolwick *et al.*, 1993). The plasmid pGST-TFIIB was obtained from Dr. Danny Reinberg (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Camden, NJ) (Ha *et al.*, 1991). The plasmids pVP16-CRF-1 and pSG424 were obtained from Dr. Christopher Bradfield (University of Wisconsin, Madison, WI). Glutathione Sepharose 4B was purchased from Pharmacia Biotech (Piscataway, NJ) and the nickel agarose (Ni-NTA) was purchased from Qiagen (Valencia, CA). The AHR and ARNT antibodies were a gift from Dr. Richard Pollenz (Medical University of South Carolina, Charleston, SC). AntiTFIIB was purchased from Promega (Madison, WI) and purified rabbit IgG was purchased from Sigma (St. Louis, MO).

General procedures. Standard reaction mixtures for all PCR experiments were: 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each deoxyribonucleotide triphosphate, and 2.5 units *Pfu* DNA polymerase that is derived from *Pyrococcus furiosus* in a total volume of 100 μl. The PCR reactions were generally performed using annealing temperatures that were 4° below the calculated melting point of the primers. The amplified products were purified after agarose gel electrophoresis (0.8%) and electroelution and were subcloned using standard molecular biology procedures. Sequencing was performed using the dideoxy chain termination method (Sanger *et al.*, 1977).

Plasmid construction. pSportTFIIB was generated after amplification from pGST-TFIIB using HIS 51 (forward) and HIS 50 (reverse) and insertion of the product into the *Spe*I and *Hin*dIII sites of the pSport expression vector (Gibco BRL). To generate the pVP16GAL4 plasmid, the *Bam*HI/*Bgl*II fragment of pVP16-CRF-1 was inserted into the *Bam*HI site of pSG424. The VP16GAL4 fusion was amplified using the primers HIS 39 and HIS 40 and subcloned into the *Spe*I site of pSport. The following AHR constructs were generated using PCR and using pmuAHR as the template: $AHR_{(81–289)}$ was generated using the primers HIS 34 and HIS 123 and subcloning the products into the *Spe*I and *Hin*dIII sites of the pSport expression vector. $AHR_{(183–805)}$ was constructed using HIS 68 and HIS 130 as the primers and subcloning the PCR product into the *Spe*I, *Not*I site of pmuAHR. Similarly, $AHR_{(290–805)}$ was constructed using the primers HIS 88 and HIS 130 and subcloning the product into the *Spe*I/*Not*I site of pmuAHR. The $AHR_{(1–42)}$ GAL4 fusion construct was generated using the primers HIS 61 and HIS 62 and subcloning the product into the *Bam*HI site of pSG424. The fusion construct was amplified using the primers HIS 39 and HIS 40 and the product was inserted into the *Spe*I site of pSport.

Protein expression. *In vitro* expression of all AHR, ARNT, VP16GAL4, and TFIIB constructs was performed using rabbit reticulocyte lysates (Promega) as described previously (Dolwick *et al.*, 1993). For verification of protein expression, the translation reactions were performed in the presence of [^{35}S]methionine, and the products were analyzed by SDS-polyacrylamide gel electrophoresis. Quantification of the expressed proteins was determined by excising the radiolabeled proteins from the gel and scintillation counting. TFIIB-GST and GST were generated and purified from *Escherichia coli* as described previously (Ha *et al.*, 1991).

Coaffinity precipitation analysis. Coaffinity precipitation analysis was performed essentially as described previously (Swanson and Yang, 1996). Briefly, TFIIB-GST protein that was prepared from *E. coli* and bound to glutathione Sepharose 4B was incubated with ^{35}S-labeled reticulocyte, lysate-expressed AHR or ARNT in mild wash buffer (50 mM Tris, pH 7.4, 100 mM KCl, 10% glycerol, 10 mM β-mercaptoethanol, 0.4% Tween 20) for 2 hr at 4° with gentle mixing. As a negative control, the ^{35}S-labeled proteins were incubated with only the GST bound Sepharose 4B. The Sepharose 4B was washed

five times using wash buffer and pelleted after centrifugation at 16,000 × *g* for 10 sec. Sample buffer was added to each pellet, the mixture was boiled and the eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Coaffinity precipitation assays to detect an interaction between the ^{35}S-labeled proteins and six histidine-tagged AHR or ARNT were performed similarly except that the wash buffer included 5 mM imidazole (Swanson and Yang, 1996). Quantification of the radiolabeled bands was performed after PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis.

Gel shift analysis. The DNA probe (annealed HIS 17/18) containing the DRE was radiolabeled with [γ-^{32}P]ATP by endlabeling with T4 polynucleotide kinase (Garabedian *et al.*, 1993). For these experiments, we used the AHR variant protein [AHR$_{(1–289)}$] that lacks 516 amino acids from the carboxyl terminus and interacts with ARNT in a ligand-independent manner (Dolwick *et al.*, 1993). Reticulocyte-expressed AHR and ARNT proteins were incubated for 30 min at 30° to aid dimerization. In some cases, reticulocyte expressed TFIIB was incubated with the AHR-containing mixtures either before or after incubation with ARNT. Two hundred nanograms of nonspecific competitor [poly(dI-dC)] was added, the KCl concentration was adjusted to 100 mM, and the mixture was incubated at room temperature for 10 min. The radiolabeled probe (100,000 cpm; 0.5 ng) was added, the mixture was incubated for 10 min and was analyzed by nondenaturing gel electrophoresis using 0.5 × Tris/borate/EDTA (45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0) as the running buffer (Garabedian *et al.*, 1993). The gels were dried and the radiolabeled bands were quantified by PhorphorImager analysis.

Results

TFIIB interacts with the AHR and ARNT. To determine whether TFIIB interacts with either the AHR or ARNT, we used the coaffinity precipitation assay. The radiolabeled ARNT or AHR proteins were incubated with the TFIIB-GST fusion protein that was immobilized to glutathione Sepharose 4B beads. After several washings, the beads were collected by centrifugation and the ^{35}S-labeled proteins that interact with TFIIB were seen after SDS-gel electrophoresis and autoradiography. To verify that the detected radiolabeled bands represented specific interactions with TFIIB, we performed identical experiments except using only immobilized GST. As shown in Fig. 1A, *lanes 1*, *4* and *7*, a limited amount of ^{35}S-labeled ARNT was detected after incubation of ^{35}S-labeled ARNT with the TFIIB-GST fusion protein. Similarly, incubations that were performed using ^{35}S-labeled AHR, resulted in the detection of a radiolabeled band when the AHR was incubated with the TFIIB-GST fusion protein (Fig. 1A, *lanes 2* and *5*) but not GST alone (Fig. 1A, *lane 8*). These results indicate that both the AHR and ARNT are capable of interacting with TFIIB. As shown in Fig. 1A, *in vitro* transcription/translation of both the AHR and ARNT proteins results in the appearance of several lower molecular mass bands that probably represent transcriptional pauses. Interestingly, several of these bands that represent smaller fragments of both the AHR and ARNT also interact with TFIIB.

To ensure that our conditions were appropriate for detecting protein/protein interactions with TFIIB, we performed similar incubations, except that we used a ^{35}S-labeled fusion protein composed of the activation domain of VP16 and the amino-terminal 147 amino acids of the yeast GAL4 protein (Fig. 1A, *lanes 3*, *6* and *9*). The interactions of this VP16 fragment with TFIIB have been well characterized (Lin and Green, 1991) and serve as a positive control.

To verify that both the AHR and ARNT interact with TFIIB, similar coaffinity precipitation experiments were performed, except that we used either immobilized AHR or ARNT and ^{35}S-TFIIB (Fig. 1B). Using these conditions, TFIIB was found to interact with both the AHR (Fig. 1B, *lanes 1* and *2*) and ARNT (Fig. 1B, *lanes 1* and *3*). Thus, use of high concentrations of ARNT (approximately 40 μg) allows the TFIIB/ARNT interaction to be detected easily. Specificity of the TFIIB interaction was demonstrated by the absence of the radiolabeled 33-kDa band when ^{35}S-TFIIB was incubated with only the agarose matrix (Fig. 1B, *lane 4*). To confirm that the overexpressed ARNT protein is capable of forming protein/protein interactions, we incubated ^{35}S-AHR with the histidine-tagged ARNT and performed coaffinity precipitation assays. As shown in Fig. 1C, *lanes 1*, *2* and *3*, the ^{35}S-AHR specifically interacts with the immobilized ARNT.

TFIIB interacts with the PAS and carboxyl-terminal regions of the AHR. Once we had determined that TFIIB interacts with the AHR, our next goal was to determine the region within the AHR that facilitates its interaction with TFIIB. To achieve this goal, we generated variant ^{35}S-labeled AHR proteins that represented either carboxyl-terminal deletions, amino-terminal deletions, or fusion proteins of the AHR (Fig. 2A) and performed coaffinity precipitation assays using the immobilized TFIIB-GST fusion protein. Our initial experiments indicate that deletion of the basic region but not the carboxyl-terminal region of the AHR abolished its ability to interact with TFIIB (Fig. 2B). These results indicate that the basic region of the AHR may not be involved in mediating the TFIIB/AHR interaction. Although it is possible that fusion of the GAL4 protein to the basic region of the AHR hinders an interaction with TFIIB, this scenario is unlikely, because a similar fusion of the VP16 activation domain with GAL4 permits the VP16/TFIIB interaction to occur (Fig. 1, Fig. 2C, *lanes 1–3*). Further, the ability of the AHR$_{(81–289)}$ construct (Fig. 2C, *lanes 4–6*) but not the AHR$_{(183–805)}$ construct (Fig. 2C, *lanes 7–9*) to interact with TFIIB indicates that a primary site of the AHR/TFIIB interaction occurs with the PAS region of the AHR and lies within the region bordered by amino acids 81 and 183. Interestingly, further amino-terminal deletions of the AHR represented by the AHR$_{(290–805)}$ construct resulted in an interaction between AHR and TFIIB, indicating that a secondary site of interaction with TFIIB occurs within the carboxyl terminus of the AHR (Fig. 2C, *lanes 10–12*).

Similar to the results shown in Fig. 1A, a number of lower molecular mass bands that are generated by *in vitro* transcription/translation of the full length AHR (Fig. 2B, *lanes 1–3*) and the AHR$_{(183–805)}$ and the AHR$_{(290–805)}$ constructs (Fig. 2C, *lanes 7–12*) interact with TFIIB. In addition, although these assays are not quantitative in nature, it seems that the deletion constructs AHR$_{(1–289)}$ (Fig. 2B, *lanes 4–6*) and AHR$_{(81–289)}$ (Fig. 2C, *lanes 4–6*) are pulled down with greater efficiency than that observed using the full length AHR (Fig. 2B, *lanes 1–3*). Given these observations, together with the observation that AHR$_{(290–805)}$, but not AHR$_{(183–805)}$ interacts with TFIIB (Fig. 2C, *lanes 7–12*), we suggest that a region that is bordered by amino acids 183 and 290 may act to repress the AHR/TFIIB interaction.

The fact that TFIIB interacts with the PAS domain of the

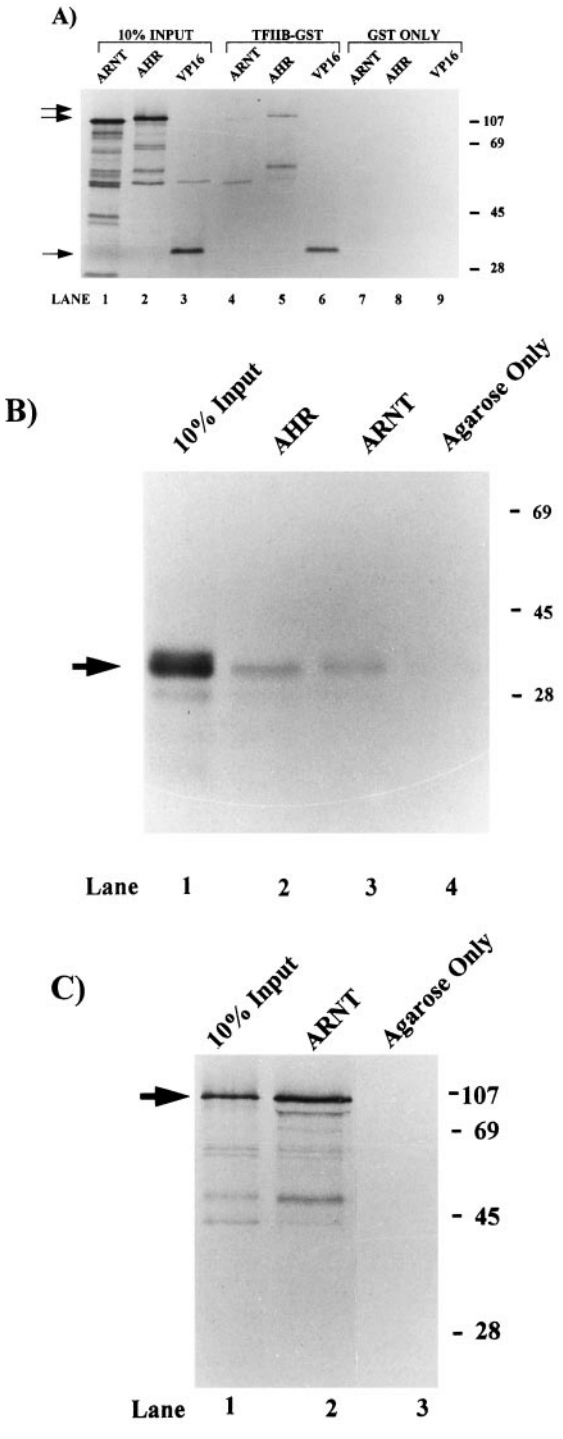

Fig. 1. TFIIB interacts with the AHR and to a lesser extent with ARNT. Protein-protein interactions were analyzed using the coaffinity precipitation assay. *Right*, migration of the indicated molecular mass markers (kDa). A, ^{35}S-labeled AHR and ARNT interact with TFIIB. *Lanes 1–3*, 10% of the input proteins. Equal concentrations of the indicated ^{35}S-labeled proteins were incubated with either the fusion protein TFIIB-GST (*lanes 4–6*) or GST alone (*lanes 7–9*) immobilized on Sepharose 4B beads as described in Experimental Procedures. After several washes, the pellet was applied to a SDS-polyacrylamide electrophoretic gel and the associating proteins analyzed after autoradiography. *Arrows on left*, ARNT, AHR, or VP16GAL4 radiolabeled bands. B, ^{35}S-labeled TFIIB interacts with both the six-histidine-tagged AHR and the six-histidine-tagged ARNT. Coaffinity precipitation analysis was performed as described in Experimental Procedures using ^{35}S-labeled TFIIB (0.5 fmol) and approximately 40 μg of either baculovirus-expressed AHR (*lane 2*), ARNT (*lane 3*), or the nickel agarose alone (*lane 4*). *Arrow on left*, TFIIB radiolabeled band. C, Six-histidine-tagged ARNT interacts with the AHR. ^{35}S-labeled AHR (*lane 1*) was incubated with either 40 μg of baculovirus expressed ARNT (*lane 2*) or the nickel agarose alone (*lane 3*) and the reactions analyzed using the coaffinity precipitation assay as described above with the addition of 10 μM β-naphthoflavone. *Arrow on left*, AHR radiolabeled band.

AHR that also mediates ligand binding, Hsp90 association, and dimerization with ARNT (Dolwick *et al.*, 1993; Reisz-Porszasz *et al.*, 1994; Lindebro *et al.*, 1995) led us to question whether ligand activation of the AHR or heterodimerization with ARNT would affect the interaction between the AHR and TFIIB. To address this question, we performed additional coaffinity precipitation assays. Simultaneous incubations of the AHR with either the agonist β-naphthoflavone or the partial antagonist α-naphthoflavone did not affect the ability of the full length AHR protein to interact with TFIIB (data not shown) indicating that ligand activation of the AHR and the subsequent dissociation of Hsp90 is not required to facilitate the AHR/TFIIB interaction.

The interaction between TFIIB and the AHR enhances the ability of the AHR/ARNT complex to bind DNA. In an effort to determine whether TFIIB affects formation of the AHR/ARNT complex at concentrations that are more representative of those that may occur at the physiological levels within the cell, we performed gel shift analysis. Gel shift assays were performed in the presence of increasing concentrations of reticulocyte expressed TFIIB. As shown in Fig. 3A, preincubation of the AHR alone, before incubation with ARNT, compromises DNA binding of the AHR/ARNT complex (Fig. 3A, *lanes 1* and *5*) suggesting that the DNA binding form of the AHR protein is stabilized by the presence of its DNA binding partner, ARNT. The ability of TFIIB to partially fulfill this stabilizing function in the absence of ARNT is shown in *lanes 1–4*. Increasing amounts of TFIIB that were incubated with the AHR before its dimerization with ARNT enhanced DNA binding of the AHR/ARNT heterodimer. Quantification of three independent experiments demonstrated that the addition of 3 fmol of TFIIB resulted in a 186 $\pm$ 20-fold increase in AHR/ARNT complex formation. However, when similar amounts of TFIIB were added to the preformed AHR/ARNT complex, DNA binding of the AHR/ARNT heterodimer was unaffected (Fig. 3A, compare *lane 5* with *lanes 6–8*). The fact that the observed complex is composed of the AHR and ARNT is demonstrated by the ability of antibodies that recognized either the AHR (Fig. 3A, lane 9) or ARNT (Fig. 3A, *lane 10*) but not nonspecific IgG (Fig. 3A, *lane 11*) to eliminate formation of the protein/DNA complex.

To provide further evidence that TFIIB mediates an increase in AHR/ARNT DNA binding, we performed similar incubations in the absence or presence of a TFIIB antibody. As shown in Fig. 3B, *lanes 1–5*, the increased DNA binding observed with the addition of TFIIB to the AHR (before the addition of ARNT) was abrogated with the coincubation of the TFIIB antibody. Specificity of the TFIIB antibody is demonstrated by performing a similar incubation except using purified IgG (Fig. 3B, *lane 6*). Finally, the inability of the TFIIB antibody to alter the mobility of the AHR/ARNT complex when added to the protein mixture after the addition of DNA indicates that TFIIB does not interact with the DNA bound form of the AHR (Fig. 3B, *lane 7*). The consistency of the rate at which the AHR/ARNT complex migrates after gel shift analysis, together with the use of antibodies that rec-

ognize the AHR, ARNT, and TFIIB, indicates that the TFIIB augmented band contains both the AHR and ARNT proteins.

Discussion

The initiation of transcription by RNA polymerase II in eukaryotes includes proper assembly of a complex of proteins that includes the general transcription factors TFIIA-B, D, -E, -F, and -H (Goodrich *et al.*, 1996). Protein-protein contacts between the general transcription factors and activator proteins are thought to facilitate transcriptional activation and have been described between TFIID, TFIIF, TFIIH, and TFIIB and a number of activator proteins (Zawel and Reinberg, 1995). In this manner, a general transcription factor may form a bridge between the activator protein and RNA polymerase II. For example, TFIIB participates in interactions with RNA polymerase II, the TATA box binding protein and a number of activator proteins. In an effort to obtain a better understanding of gene activation by the b/HLH/PAS family of transcription factors, we questioned whether either the AHR or ARNT interacts directly with TFIIB. In this study, we have demonstrated that both the AHR and ARNT interact with TFIIB.

The interactions between TFIIB and activator proteins are diverse in that TFIIB interacts with several different types of functional domains of the activator protein and that the TFIIB/activator protein interaction seems to trigger distinct events. Although initial studies that characterized the interaction between TFIIB and VP16 implied that TFIIB interacts with only the activation domains of activator proteins (Lin and Green, 1991), subsequent work has demonstrated that TFIIB may also interact with the ligand binding domains of the thyroid hormone receptor and the vitamin D receptor (Baniahmad *et al.*, 1993; MacDonald *et al.*, 1995). In fact,

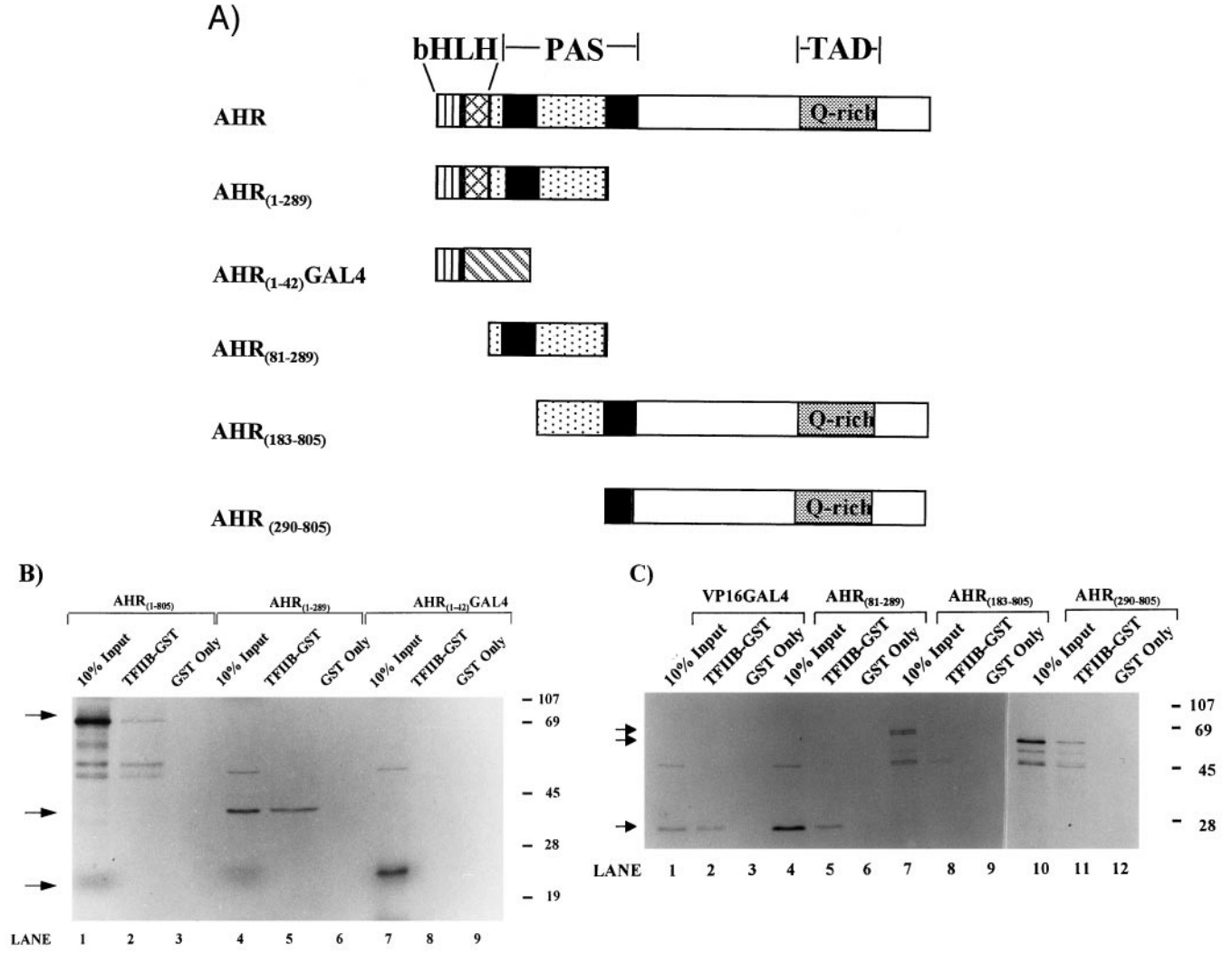

Fig. 2. TFIIB interacts with the PAS and carboxyl-terminal regions of the AHR. A, Schematic representation of variant AHR proteins. ▥, basic region; ▩, helix-loop-helix region; ⊡, PAS domain; ■, A and B repeats; ▤, glutamine-rich region within the transcriptional activation domain (*TAD*); ▧, yeast Gal4 protein. B, Deletion mapping of the AHR/TFIIB interaction. *Lanes 1*, *4*, and *7*, 10% of the input protein. Either the fusion protein TFIIB-GST (*lanes 2*, *5* and *8*) or GST alone (*lanes 3*, *6*, and *9*) that was immobilized on Sepharose 4B beads was incubated with the indicated ^{35}S-labeled proteins and analyzed by coaffinity precipitation as described in Experimental Procedures. After several washes, the pellets were applied to a SDS electrophoretic gel and the associating proteins were detected by autoradiography. C, TFIIB interacts with both the PAS domain and carboxyl terminus of the AHR. *Lanes 1*, *4*, *7*, and *10*, 10% of the input protein. Either the fusion protein TFIIB-GST (*lanes 2*, *5*, *8*, and *11*) or GST alone (*lanes 3*, *6*, *9*, and *12*) that was immobilized on Sepharose 4B beads was incubated with the indicated ^{35}S-labeled proteins and analyzed by coaffinity precipitation as described above. *Arrows on left*, appropriate AHR constructs; *right*, migration of the indicated molecular mass markers (kDa).

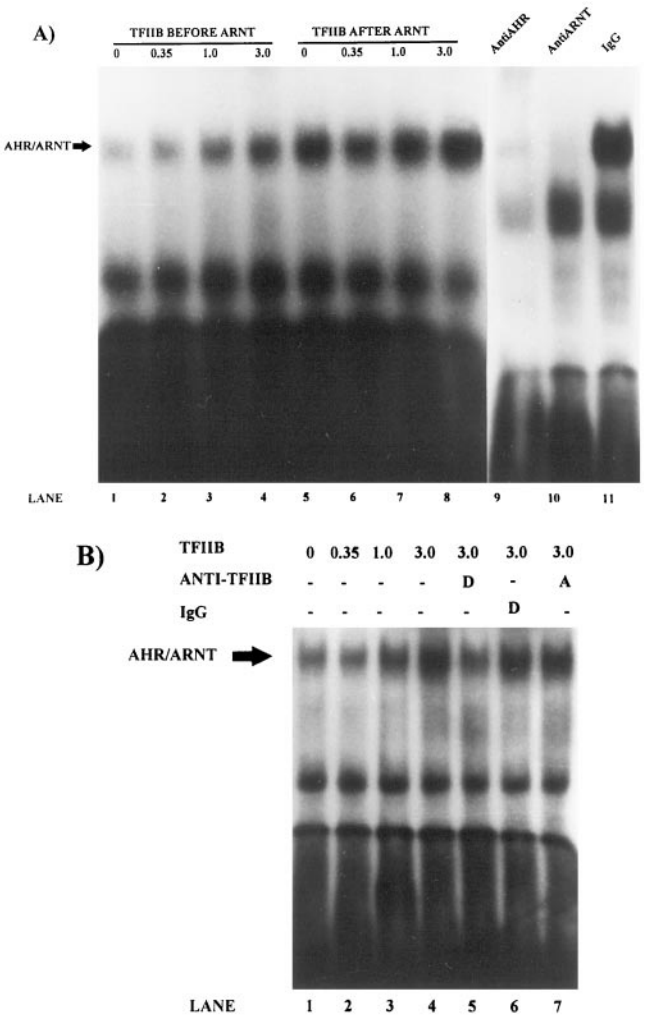

Fig. 3. Gel shift analysis demonstrates that the interaction of TFIIB with the PAS region of the AHR enhances the ability of the AHR/ARNT complex to bind DNA. The protein mixtures were incubated as described below and analyzed by the gel shift assay using annealed HIS17/18 oligonucleotides as the probe. The AHR protein used in these experiments is the $AHR_{(1-289)}$ variant protein that lacks the carboxyl-terminal 516 amino acids and interacts with ARNT in a ligand-independent manner (Dolwick *et al.*, 1993). The protein concentrations in each sample were normalized after the addition of unprogrammed reticulocyte lysate extracts. A, Increasing concentrations of TFIIB result in a corresponding increase in DNA binding of the AHR/ARNT complex. *Lanes 1–4*, the indicated concentrations (fmol) of reticulocyte expressed TFIIB were incubated with 0.2 fmol of reticulocyte $AHR_{(1-289)}$ for 2 hr at 4°, 0.2 fmol of reticulocyte ARNT was added and the protein mixture incubated for an additional 30 min at 30°. *Lanes 5–8*, equal concentrations of reticulocyte expressed $AHR_{(1-289)}$ and ARNT (0.2 fmol) were incubated for 30 min at 30°. The indicated concentrations of TFIIB were added and the protein mixture was incubated for 2 hr at 4°. *Lanes 9–11*, reticulocyte expressed $AHR_{(1-289)}$ and ARNT (0.2 fmol each) were incubated for 30 min at 30°. The supershift experiments were performed (*lanes 9–11*) by the addition of 0.2 μg of either antiAHR, antiARNT, or nonspecific immunoglobulins to the protein/DNA mixture and incubating further incubating the mixture at room temperature for 10 min after the addition of the probe. B, The increase in AHR/ARNT binding is specific to the addition of TFIIB. *Lanes 1–4*, increasing concentrations of reticulocyte TFIIB was incubated with $AHR_{(1-289)}$ before the addition of ARNT as described in A. The TFIIB antibody was added either during (*D*) or after (*A*) the incubation of reticulocyte TFIIB with the $AHR_{(1-289)}$ protein. Reticulocyte expressed TFIIB, the $AHR_{(1-289)}$ and 0.2 μg of either antiTFIIB (*lane 5*) or purified IgG (*lane 6*) was incubated for 2 hr at 4° before the addition of ARNT. *Lane 7*, reticulocyte TFIIB was incubated with the $AHR_{(1-289)}$ before the addition of ARNT as described in part A and 0.2 μg of antiTFIIB was added to the protein mixture after the addition of the ^{32}P-labeled probe and before nondenaturing gel electrophoresis.

TFIIB interacts with both the amino-terminal and carboxyl-terminal regions of the human thyroid receptor β (Baniahmad *et al.*, 1993). Similarly, we have shown that TFIIB interacts with two regions of the AHR (Fig. 2). Once TFIIB interacts with the activator protein, it may increase the stable assembly of the preinitiation complex (e.g., VP16) (Lin and Green, 1991; Roberts *et al.*, 1993), facilitate a silencing activity (thyroid receptor) or increase the ability of the activator proteins to bind DNA (IRF, Oct 1, the AHR) (Nakshatri *et al.*, 1995; Wang *et al.*, 1996; Fig. 3) Alternatively, the formation of an activator protein/TFIIB complex may have little or no effect on the ability of the activator protein to enhance gene transcription (nuclear factor κB65) (Schmitz *et al.*, 1995). Thus, although TFIIB may interact with a variety of transcription factors, the events initiated by this interaction seem to be dependent on the interacting activator protein.

The PAS domain of the AHR mediates a number of diverse events that includes ligand binding, dimerization with its DNA binding partner, and associations with proteins such as Hsp90, SP1, and TFIIB (Dolwick *et al.*, 1993; Reisz-Porszasz *et al.*, 1994; Coumailleau *et al.*, 1995; Lindebro *et al.*, 1995; Kobayashi *et al.*, 1996; Fig. 2). Although the PAS domain of the AHR and ARNT represents a somewhat poorly conserved domain that does not seem to represent a definitive structural motif, its hallmark consists of two direct repeats, the A and B repeats. The amino-terminal and carboxyl-terminal halves of the PAS domain, together with its helix-loop-helix region, mediates dimerization with its DNA binding partner, ARNT (Dolwick *et al.*, 1993; Reisz-Porszasz *et al.*, 1994; Lindebro *et al.*, 1995). In fact, the primary interaction between the AHR and ARNT seems to map to the carboxyl-terminal half (the B repeat, amino acids 230–421) of the AHR PAS domain. Within this region lies the ligand binding domain and a site for an Hsp90 interaction (Lindebro *et al.*, 1995). In the present study, we have shown that TFIIB interacts with the AHR at a site (the region bordered by amino acids 81 and 183) that lies slightly adjacent to its ARNT dimerization motif as well as interacting with its carboxyl terminus. The ability of the PAS domain of the AHR to interact with both SP1 (Kobayashi *et al.*, 1996) and TFIIB (Fig. 2) implies that this region, in addition to the carboxyl-terminal region, is an important contributor to transcriptional activation by the AHR/ARNT complex.

Previous studies examining the transcriptional activating potencies of the AHR and ARNT as well as their associations with other transcription factors have indicated that the AHR and ARNT may play distinct roles during the activation process (Jain *et al.*, 1994; Ko *et al.*, 1996; Rowlands *et al.*, 1996). A comparison between the ability of individual AHR or ARNT proteins to activate transcription has revealed that the activating potencies of the AHR and ARNT are similar (Jain *et al.*, 1994). However, when analyzed within the context of the AHR/ARNT complex, only that of the AHR is functional during dioxin-induced transcription of CYP1A1. Thus, it seems that only the AHR facilitates the communication from the GCGTG enhancer to the proximal promoter region (Ko *et al.*, 1996), which implies that the AHR, but not ARNT, participates in the crucial protein/protein interactions that facilitate transcriptional activation. This idea is supported by the observation that the AHR, but not ARNT, interacts with the TATA binding protein (Rowlands *et al.*,

1996). It remains to be determined how the interaction of either the AHR or ARNT with TFIIB may influence the ability of either of these proteins to activate genes.

Our results indicate that TFIIB may assist in the formation of the DNA binding complex of the AHR/ARNT heterodimer by stabilizing the AHR before its interaction with ARNT. This idea is supported by the data presented in Fig. 3. The addition of increasing concentrations of TFIIB resulted in a corresponding increase in DNA binding of the AHR/ARNT complex only if TFIIB was first incubated with the AHR. However, TFIIB did not affect DNA binding of the AHR/ARNT complex when it was added after AHR/ARNT heterodimerization. In summary, these results suggest a novel role for TFIIB. In addition to its role in recruiting transcription factors to the TATA box, it may also assist in the heterodimerization of activator proteins such as the AHR and ARNT.

Acknowledgments

We thank Danny Reinberg for the human TFIIB cDNA, Richard Pollenz for the antibodies to the AHR and ARNT proteins, and Dr. Christopher Bradfield for the pmiAHR, pmuAHRCΔ516, VP16-CRF and pSG424 plasmids.

References

Baniahmad A, Ha I, Reinberg D, Tsai S, Tsai M-J, and O'Malley BW (1993) Interaction of human thyroid hormone receptor β with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. *Proc Natl Acad Sci USA* **90:**8832–8836.

Blanco JCG, Wang I-M, Tsai SY, Tsai M-J, O'Malley BW, Jurutka PW, Haussler MR, and Ozato K (1995) Transcription factor IIB and the vitamin D receptor cooperatively activate ligand-dependent transcription. *Proc Natl Acad Sci USA* **92:**1535–1539.

Burbach KM, Poland A, Bradfield CA (1992) Cloning of the Ah receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci USA* **89:**8185–8189.

Chen H, Chrast R, Rossier C, Gos A, Antondrakis SE, Koduh J, Yamaki A, Shindoh N, Maeda H, Minoshima S, and Shimizu N (1995) Single-minded and Down syndrome? *Nat Genet* **10:**9–10.

Coumailleau P, Poellinger L, Gustafsson J-A, and Whitelaw ML (1995) Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild- type ligand binding affinity and specificity. *J Biol Chem* **270:**25291–25300.

Dolwick KM, Swanson HI, and Bradfield CA (1993) *In vitro* analysis of the Ah receptor domains involved in ligand-activated DNA recognition. *Proc Natl Acad Sci USA* **90:**8566–8570.

Garabedian MJ, LaBaer J, Liu W-H, and Thomas JR (1993) Analysis of protein-DNA interactions, in *Gene Transcription: A Practical Approach* (Hames BD and Higgins SJ, eds) pp 243–293, Oxford University Press, New York, NY.

Goodrich JA, Cutler G, and Tjian R (1996) Contacts in context: promoter specificity and macromolecular interactions in transcription. *Cell* **84:**825–830.

Ha I, Lane WS, and Rinberg D (1991) Cloning of a human gene encoding the general transcription initiation factor IIB. *Nature (Lond)* **352:**689–695.

Hoffman EC, Reyes H, Chu FF, Sander F, Conley LH, Brooks BA, and Hankinson O (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science (Washington DC)* **252:**954–958.

Hogenesch JB, Chan WK, Jackiw VH, Brown RC, Gu Y-Z, Pray-Grant M, Perdew GH, and Bradfield CA (1997) Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J Biol Chem* **272:**8581–8593.

Hong H, Kohli K, Garabedian MJ, and Stallcup MR (1997) GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptor. *Mol Cell Biol* **17:**2735–2744.

Ing NH, Beekman JM, Tsai SY, Tsai M-J, and O'Malley BW (1992) Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). *J Biol Chem* **267:**17617–17623.

Jackson FR, Bargiello TA, Yun SH, and Young MW (1986) Product of the per locus of *Drosophila* shares homology with proteoglycans. *Nature (Lond)* **320:**185–88.

Jain S, Dolwick KM, Schmidt J, and Bradfield CA (1994) Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxy termini. *J Biol Chem* **269:**31518–31524.

Ko HP, Okino ST, Ma Q, and Whitlock JP Jr (1996) Dioxin-induced CYP1A1 transcription in vivo: the aromatic hydrocarbon receptor mediates transactivation, enhancer-promoter communication, and changes in chromatin structure. *Mol Cell Biol* **16:**430–436.

Kobayashi A, Sogawa K, and Fujii-Kuriyama Y (1996) Cooperative interaction between AhR-Arnt and Sp1 for the drug-inducible expression of CYP1A1 gene. *J Biol Chem* **271:**12310–12316.

Konopka RJ and Benzer S (1971) Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **68:**2112–2116.

Li H, Gomes P, and Chen JD (1997) RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci USA* **94:**8479–8484.

Lin Y-S and Green MR (1991) Mechanism of action of an acidic transcriptional activator *in vitro*. *Cell* **64:**971–981.

Lindebro MC, Poellinger L, and Whitelaw ML (1995) Protein-protein interactions via PAS domains: role of the PAS domains in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *Eur Mol Biol Org J* **14:**3528–3539.

Ma Q, Dong L, and Whitlock JP Jr (1995) Transcriptional activation by the mouse Ah receptor. *J Biol Chem* **270:**12697–12703.

MacDonald PN, Sherman DR, Dowd DR, Jefcoat SC Jr, and DeLisle RK (1995) The vitamin D receptor interacts with general transcription factor IIB. *J Biol Chem* **270:**4748–4752.

Muralidhar MG, Callahan CA, Thomas JB (1993) Single-minded regulation of genes in the embryonic midline of the *Drosophila* central nervous system. *Mech Dev* **41:**129–138.

Nambu JR, Lewis JO, Wharton KA Jr, and Crews ST (1991) The *Drosophila* single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67:**1157–1167.

Nakshatri H, Nakshatri P, and Currie RA (1995) Interaction of Oct-1 with TFIIB. *J Biol Chem* **270:**19613–19623.

Onate SA, Tsai SY, Tsai M-J, and O'Malley BW (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science (Washington DC)* **270:**1354–1357.

Reisz-Porszasz S, Probst MR, Fukunaga BN, and Hankinson O (1994) Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Mol Cell Biol* **14:**6075–6086.

Roberts SGE, Ha I, Maldonado E, Reinberg D, and Green MR (1993) Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature (Lond.)* **363:**741–747.

Rowlands JC, McEwan IJ, and Gustafsson J-A (1996)*Trans*-activation by the human aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator proteins: direct interactions with basal transcription factors. *Mol Pharmacol* **50:**538–548.

Sanger F, Nicklen S, and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:**5463–5467.

Schmitz ML, Stelzer G, Atlmann H, Meisterernst M, and Baeuerle PA (1995) Interaction of the COOH-terminal transactivation domain of p64 NF-κB with TATA binding protein, transcription factor IIB and coactivators. *J Biol Chem* **270:**7219–7226.

Swanson HI and Bradfield CA (1993) The AH-receptor: genetics, structure and function. *Pharmacogenetics* **3:**213–230.

Swanson HI, Chan WK, and Bradfield CA (1995) DNA binding and pairing rules of the Ah receptor, ARNT and SIM proteins. *J Biol Chem* **270:**26292–26302.

Swanson HI and Yang J-H (1996) Mapping the protein/DNA contact sites of the Ah receptor and Ah receptor nuclear translocator. *J Biol Chem* **271:**31657–31665.

Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, and Sakaki Y (1997) Circadian oscillation of mammalian homologue of the *Drosophila* period gene. *Nature (Lond)* **389:**512–516.

Vitaterna MH, King DP, Chang A-M, Kornhauser JM, Lowrey PL, McDonald JD, Dove JM, Pinto LH, Turek FW, and Takahashi JS (1994) Mutagenesis and mapping of a mouse gene, clock, essential for circadian behavior. *Science (Washington DC)* **264:**719–725.

Voegel JJ, Heine MJS, Zechel C, Chambon P, and Gronemeyer H (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO (Eur Mol Biol Organ) J* **15:**3667–3675.

Wang GL, Jiang B-H, Rue EA, and Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* **92:**5510–5514.

Wang I-M, Blanco JCG, Tsai SY, Tsai M-J, and Ozato K (1996) Interferon regulatory factors and TFIIB cooperatively regulate interferon-responsive promoter activity *in vivo* and *in vitro*. *Mol Cell Biol* **16:**6313–6324.

Zawel L and Reinberg D (1995) Common themes in assembly and function of eukaryotic transcription complexes. *Ann Rev Biochem* **64:**533–561.

Send reprint requests to: Dr. Hollie I. Swanson, Department of Pharmacology, 800 Rose Street, MS311 UKMC, University of Kentucky, Lexington, KY 40536. E-mail: hswan@pop.uky.edu