

Endogenous Hepatic Expression of the Hepatitis B Virus X-Associated Protein 2 Is Adequate for Maximal Association with Aryl Hydrocarbon Receptor-90-kDa Heat Shock Protein Complexes

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that acts as an environmental sensor by binding to a variety of xenobiotics. AHR activation serves to combat xenotoxic stress by inducing metabolic enzyme expression in the liver. The hepatitis B virus X-associated protein (XAP2) is a component of the cytosolic AHR complex and modulates AHR transcriptional properties in vitro and in cell culture and yeast systems. Expression of XAP2 is low in liver compared with other nonhepatic tissues and the AHR exhibits high ligand-induced transcriptional activity. Because XAP2 has been demonstrated to repress AHR activity, we hypothesized that XAP2 may be limiting in liver and that increasing XAP2 levels would attenuate AHR transcriptional activity. To this end, transgenic mice were generated that exhibit hepatocyte-specific elevation in XAP2 expression. Transgenic XAP2 expression was restricted to liver, and its ability to complex with the AHR was

verified. Gene expression experiments were performed by inducing AHR transcriptional activity with β -naphthoflavone via intraperitoneal injection, and mRNA quantification was done by real-time polymerase chain reaction. Wild-type and transgenic animals showed little difference in constitutive or ligand-induced CYP1A1; CYP1A2; UDP glucuronosyltransferase 1A2; NAD(P)H dehydrogenase, quinone 1; constitutive androstane receptor; or nuclear factor erythroid 2-related factor 2 mRNA expression. Sucrose density fractionation and AHR immunoprecipitation experiments found little or no stoichiometric increase in bound XAP2 to the AHR between genotypes. Gene array studies were performed to identify novel XAP2-regulated targets. Taken together, this work shows that despite the relatively low level of XAP2 in liver, it is not a limiting component in AHR regulation.

The aryl hydrocarbon receptor (AHR) is a soluble, ligand-activated transcription factor that mediates the metabolic clearance, and at times the procarcinogenic biotransforma-

tion, of xenobiotics and endogenous compounds through the transcriptional induction of phase I and II metabolic enzymes such as the cytochromes P450 1A1, 1A2, and 1B1 as well as UDP glucuronosyltransferase, NAD(P)H:oxidoreductase, and glutathione transferase Ya subunit (for review, see Nebert et al., 2000). In rodent models, numerous toxic effects have been observed to be mediated through AHR activation, including cancer, liver toxicity, thymic atrophy, and teratogenesis. These effects are elicited through exposure to the high affinity and metabolically resistant AHR ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and, to some extent,

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ABBREVIATIONS: AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HSP90, 90-kDa heat shock protein; XAP2, hepatitis B virus X-associated protein 2; PCR, polymerase chain reaction; QRT-PCR, quantitative real-time polymerase chain reaction; β -NF, β -naphthoflavone; FABPL, liver fatty acid binding protein; ACOX1, acyl-coenzyme A oxidase 1; PBS, phosphate-buffered saline; NQO1, NAD(P)H dehydrogenase, quinone 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UGT1A2, UDP-glucuronosyltransferase 1A2; CAR, constitutive androstane receptor; Nrf2, nuclear factor erythroid 2-related factor 2; PPAR, peroxisome proliferator-activated receptor; Wy-14,643, pirinixic acid; 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; MENG, 25 mM 3-(*N*-morpholino)propanesulfonic acid, 2 mM EDTA, 0.02% Na₂S₂O₃, and 10% glycerol, pH 7.5; PVDF, polyvinylidene difluoride; TSDS-PAGE, Tricine SDS-polyacrylamide gel electrophoresis; RXR, retinoid X receptor; NP-40, Nonidet P-40; PER/ARNT/SIM, periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded.

polycyclic aromatic hydrocarbons such as benzo[a]pyrene. The generation of AHR null mouse strains (Fernandez-Salguero et al., 1995; Schmidt et al., 1996) has established that most of the deleterious effects observed through TCDD exposure are mediated through the AHR, because these animals are refractory to TCDD-induced toxicity.

The AHR is a member of the basic helix-loop-helix PER/ARNT/SIM family of transcription factors. Members of the basic helix-loop-helix PER/ARNT/SIM family tend to be involved in maintaining homeostasis, such as regulating circadian rhythms (Panda et al., 2002) and alleviating hypoxia (Kewley et al., 2004). Although the physiological role for the AHR has yet to be fully understood, studies involving AHR null mice have revealed that the receptor is an important player in regulating pathways involved in maturation of fetal vasculature in the developing liver (Lahvis et al., 2000; Walisser et al., 2004). In addition, reduced fertility is observed in AHR null females, which has recently been suggested to be attributed to the deregulation of estradiol production through altered CYP19 gene expression in ovarian granulosa cells (Baba et al., 2005). AHR-deficient mice also display a propensity for the development of liver fibrosis because of the impaired ability to maintain retinoid homeostasis (Andreola et al., 2004). Age-dependent formation of lesions in multiple organs of AHR null mice have also been reported, implying that the AHR is necessary in maintaining homeostasis throughout life (Fernandez-Salguero et al., 1997).

The AHR, in its inactive form, exists in the cytoplasm as a complex consisting of a dimer of HSP90 (Chen and Perdew, 1994) and potentially at least one molecule of the cochaperones XAP2 (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998) and p23 (Nair et al., 1996; Kazlauskas et al., 1999). HSP90 is an essential component of the unliganded AHR complex, and its presence is necessary to maintain optimal ligand-binding capacity (Pongratz et al., 1992; Carver et al., 1994). The capability of XAP2, also known as aryl hydrocarbon receptor interacting protein (Ma and Whitlock, 1997) or Ah receptor-associated protein 9 (Carver and Bradfield, 1997), to modulate AHR function has been studied extensively in cell culture systems. Through the use of yeast and mammalian cell culture systems XAP2 has been demonstrated to regulate AHR transcriptional activity, cellular localization, and overall stability (Petrulis and Perdew, 2002). However, there are discrepancies between reports as to how XAP2 serves to modulate AHR function. For example, some studies report XAP2-mediated enhancement of AHR transcriptional output (Ma and Whitlock, 1997; Carver et al., 1998; LaPres et al., 2000), whereas others suggest that XAP2 exerts repressive properties on AHR transcriptional activity (Hollingshead et al., 2004; Pollenz and Dougherty, 2005). In addition, it has been reported that XAP2 stabilizes the AHR from proteasome-mediated degradation (Kazlauskas et al., 2000; Lees et al., 2003), whereas others maintain that XAP2 only exerts a modest influence in sustaining receptor levels (Hollingshead et al., 2004; Pollenz and Dougherty, 2005).

Previous studies have used mammalian cell culture, yeast, or in vitro systems to examine XAP2-mediated regulation of the AHR, often resulting in contradictory results. Because of this, it is necessary to study XAP2 function in the context of regulating the AHR in an in vivo setting. There is considerable variation in XAP2 expression within different tissues,

and it is particularly low in the liver (Meyer et al., 1998). The ability of the AHR to induce metabolic gene expression in the liver has been firmly established. Considering that XAP2 expression is relatively low in the liver combined with the understanding that the AHR is transcriptionally active in response to ligand, we wanted to see whether increasing the expression of XAP2 in the liver would serve to modify AHR behavior. This would allow us to elucidate whether differing amounts of XAP2 in tissues could be a mechanism of finely controlling AHR activity. To that end, transgenic mouse lines that display increased XAP2 expression in the liver under the transcriptional control of a hepatocyte-specific promoter were generated. This study describing the effects of increased XAP2 expression on AHR function are the first in vivo experiments of this nature to be performed to date.

Materials and Methods

Construction of mXAP2-FLAG Transgenic Mice. PCR primers were designed (5'-CATGGATATCGCCGCATGGCGGATCTCATCG-3' and 5'-CATGGATATCTC ACTTGTCATCGTCGCTTGTAGTCGTGGGAAAAGATGCC-3') to amplify the mouse XAP2 cDNA to incorporate the FLAG amino acid sequence on the C terminus. The PCR product contained EcoRV sites on the 5' and 3' ends of the mXAP2-FLAG PCR product. The amplified fragment was digested with EcoRV and blunt-end ligated into the StuI site of the TTR1ExV3 vector (a gift from Dr. Terry Van Dyke, University of North Carolina, Chapel Hill, NC) (Wu et al., 1996), creating the plasmid TTR1ExV3/XAP2-FLAG. The TTR1ExV3/XAP2-FLAG vector was sequenced to validate that the XAP2-FLAG sequence and orientation of insertion in the plasmid was correct. The TTR vector is under the transcriptional control of the liver-specific transthyretin promoter allowing for controlled expression of the transgene in hepatocytes.

The TTR1ExV3/XAP2-FLAG construct was digested with HindIII, and the 5.4-kilobase transgene fragment was purified and injected into C57BL/6J fertilized eggs at The Penn State Transgenic Mouse Facility (Department of Dairy and Animal Science, The Pennsylvania State University, University Park, PA). Founder mice and offspring were screened using PCR and QRT-PCR assays described below. C57BL/6J mice used to derive transgenic lines, and subsequently propagate animal colonies were from The Jackson Laboratory (Bar Harbor, ME). AHR^{-/-} mice were obtained from Dr. Christopher Bradfield (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI). Mice were maintained in a temperature-controlled facility on 12-h light/dark cycle and were given ad libitum access to food and water.

Screening of Transgenic Mice. Mice were screened using tail DNA isolated with the Wizard SV genomic DNA purification kit (Promega, Madison, WI). Presence of the XAP2-FLAG transgene in mice was determined by PCR using primers (Fig. 1B) specific for the transgene (primer set 1: 5'-CCCAGGGTGCTGGAGAGGATCGCCG-3' and 5'-GCGGAGGCTCTTGCCACTAGAGG-3'; primer set 2: 5'-GCCAGTGCAAGCTGGTGGCTCAGGAG-3' and 5'-CGTCGTCCTTGTAGTCGTGGG-3').

To genotype transgenic animals and estimate transgene copy number QRT-PCR was used (primer set: 5'-ACGCTGCACAGTGAATGAA-3' and 5'-CTCCACACAGGCAACTTGA-3'). This primer set amplifies a 104-base pair DNA fragment located within exon 2 of the XAP2 gene allowing for the amplification of the endogenous XAP2 and transgenic XAP2-FLAG genes simultaneously. QRT-PCR reactions were run using the DyNamo SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) on a MJ Research Opticon DNA engine (Bio-Rad, Hercules, CA). Genotyping was performed by using tail DNA from a hemizygous transgenic animal to generate a standard curve (50–0.05 ng). In addition, reactions using 5 ng of template

DNA were used for unknown transgenic genomic samples, and the relative amount of XAP2 was determined within each founder line. Hemizygous (XAP2-FLAG^{+/-}) and homozygous (XAP2-FLAG^{+/+}) mice were identified by an equal or 2-fold increase in XAP2 signal, respectively, compared with the hemizygous standard. Transgene copy number was estimated by examining the -fold increase in XAP2 signal in hemizygous versus wild-type mice. Transgene copy number was found to be the same (five to six copies) for each mouse line (Tg04 and Tg18) used in this study.

Gene Expression after Exposure to an AHR Ligand. The generation of CYP1A1 mRNA dose-response curves after the exposure to the AHR ligand β -naphthoflavone (β -NF) were performed in a similar manner as was used previously for monitoring aryl hydrocarbon hydroxylase activity (Niwa et al., 1975). Male wild-type (non-transgenic C57BL/6J), Tg04^{+/+}, and Tg18^{+/+} animals between 6 and 10 weeks of age were intraperitoneally injected with corn oil alone or β -NF (Sigma-Aldrich, St. Louis, MO) dissolved in corn oil at concentrations ranging from 1×10^{-3} to 2.5×10^2 mg/kg. Six hours after β -NF treatment mice were euthanized by asphyxiation under CO₂ exposure. Livers were excised, washed in ice-cold PBS, weighed, and ~50- to 100-mg pieces were taken from the left lobe and snap-frozen in liquid nitrogen. Female 10-week-old AHR^{-/-} mice were administered 50 mg/kg β -NF for 5 h. Samples were stored at -80°C until used for RNA isolation. Frozen tissues were homogenized with an Ultra Turrax T25 basic disperser (IKA Works, Inc., Wilmington, NC) in TRI Reagent (Sigma-Aldrich), and RNA was isolated as specified by the manufacturer. The ABI high-capacity cDNA archive kit (Ap-

plied Biosystems, Foster City, CA) was used to prepare cDNA from isolated tissue RNA.

Measurements of mRNA expression for all samples were performed by QRT-PCR using the DyNAmo SYBR Green qPCR kit on an MJ Research Opticon DNA engine. A common standard cDNA sample, generated from a 10 mg/kg β -NF-treated wild-type mouse, was used for all real-time PCR reactions, so that data points collected between runs could be directly compared. Individual expression values for each gene were normalized to GAPDH levels within each sample. Primer sequences used for the quantification of CYP1A1 mRNA were 5'-CTCTTCCCTGGATGCCTTCAA-3' and 5'-GGATGTGGCCCTTCTCAAATG-3'.

In addition, the expression of CYP1A2 (5'-ACATTTCCCAAGGAGCGCTGTATCT-3' and 5'-GTGATGGCCGAGTTGTTATTGGT-3'), NQO1 (5'-AGATGGCATCCAGTCTCCAT-3' and 5'-TTAGTCCCTCGGCCATTGTT-3'), UGT1A2 (5'-AAGGCTTTCTGACCACATGGA-3' and 5'-GGCAAATGTACTTCAGGACCAGAT-3'), GAPDH (5'-CATGGCCTTCCGTGTTCTCA-3' and 5'-GCGGCACGTGACATCCA-3'), CAR (5'-GGAGCGGCTGTGGAAATATTGCAT-3' and 5'-TCCA-TCTTTAGCAAAAGAGGCCCA-3'), and NRF2 (5'-AGCACTCCGTGAGTCTTCCATT-3' and 5'-TGTGCTTTAGGCGCGTTCTGTTT-3') were assessed at β -NF concentrations of 0, 20, and 250 mg/kg. Data were plotted and statistical analysis (F-test for CYP1A1 dose-response, two-way analysis of variance with Bonferroni post test for other gene expression) was performed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Gene Expression after Exposure to a PPAR α Ligand. Eight- to 10-week-old wild-type or Tg04^{+/+} males were left untreated, or they were administered the PPAR α -specific ligand Wy-14,643 (50 mg/kg) or corn oil vehicle via gavage. Seven hours after treatment, mice were euthanized by asphyxiation under CO₂ exposure. Livers were excised, washed in ice-cold PBS, and ~50- to 100-mg pieces were taken from the left lobe and snap-frozen in liquid nitrogen. RNA was isolated, and QRT-PCR performed as described above. QRT-PCR primer sets used for the PPAR α -regulated genes ACOX1 and FABPL were as follows: ACOX1 (5'-GTCGACCTTGTTCGCGC-A-3' and 5'-GGTTCCTCAGCACGGCTTG-3') and FABPL (5'-AGC-CAGGAGAACTTTGAGCCA-3' and 5'-CATGCACGATTTCTGACA-CCC-3').

Liver Cytosol and Whole Cell Extract Preparation. To prepare cytosol animals were euthanized by asphyxiation with CO₂, after which livers were removed and washed with ice-cold PBS. Tissue pieces were disrupted (100 mg/1 ml buffer) using a glass-Teflon homogenizer in a buffer composed of MENG supplemented with 20 mM Na₂MoO₄ and 1 \times protease inhibitor cocktail (Sigma-Aldrich). Cytosol was then obtained by centrifuging the crude homogenate at 100,000g for 1 h.

Whole cell extracts were prepared by homogenizing frozen tissues in radioimmunoprecipitation assay buffer using a glass-Teflon homogenizer. Homogenates were centrifuged at 21,000g for 45 min, and supernatant was collected. Protein content was determined in whole cell extracts with a detergent-compatible protein assay kit (Bio-Rad).

AHR and FLAG Immunoprecipitations. To immunoprecipitate the AHR 75 μ g (~10–20 μ l) of cytosol was mixed in 500 μ l of binding buffer (MENG, 20 mM Na₂MoO₄, 0.1% NP-40, and 1.0 mg/ml bovine serum albumin) followed by the addition of either 4 μ g of anti-AHR polyclonal IgG (BIOMOL Research Laboratories, Plymouth Meeting, PA) or nonspecific IgG (Sigma-Aldrich). Cytosol was allowed to mix with the antibody for 2 h at 4°C, after which 30 μ l of anti-rabbit IgG agarose (Sigma-Aldrich) was added to each sample. Immunoprecipitation reactions were then mixed at 4°C for 1 h followed by three washes with a buffer consisting of MENG, 20 mM Na₂MoO₄, 0.1% NP-40, and 50 mM NaCl, and finally one wash with MENG plus 20 mM Na₂MoO₄.

Immunoprecipitations of XAP2-FLAG from transgenic cytosols were performed by adding 50 μ g (~10–20 μ l) of cytosol to 750 μ l of binding buffer. That mixture was then allowed to mix with 30 μ l of

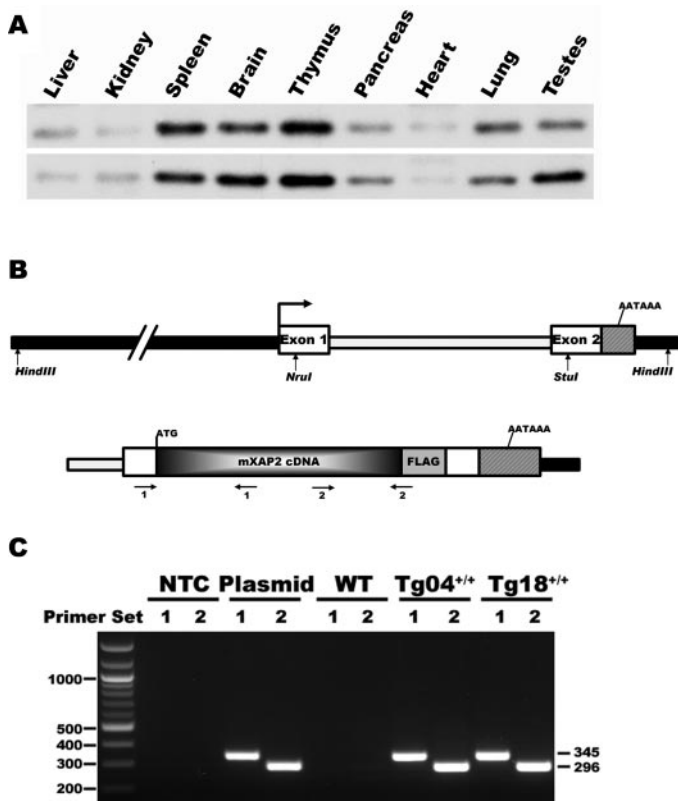


Fig. 1. Tissue distribution of XAP2 expression, and XAP2 transgenic mouse production. A, various tissues were isolated, and cytosol was prepared from two mature male C57BL/6J mice. Immunoblotting was performed to assess XAP2 levels present in these tissues. B, an illustration of the TTR1ExV3 vector used to create the transgenic mouse lines and the location (exon 2) that the XAP2-FLAG cDNA was inserted. Arrows found below the XAP2-FLAG sequence represent the location of the primer sets used to screen for transgenic animals. Note that the illustration is not drawn to scale. C, a representative PCR screen used to discriminate between transgenic and wild-type mice.

anti-FLAG M2-agarose affinity gel (Sigma-Aldrich) at 4°C for 2 h followed by three washes with the same buffer used in the AHR immunoprecipitation washes. Samples were resolved by 8% SDS-PAGE, and transferred to PVDF membrane (Millipore Corporation, Billerica, MA). Proteins were visualized by immunoblot using the following antibodies: AHR (aryl hydrocarbon receptor, RPT-1; ABR-Affinity BioReagents, Golden, CO), XAP2 (Ah receptor-associated protein, ARA-9 monoclonal; Novus Biologicals, Inc., Littleton, CO), and FLAG (anti-FLAG; ABR-Affinity BioReagents). Biotin-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in conjunction with ¹²⁵I-streptavidin (Amersham Biosciences, Piscataway, NJ) were used and autoradiograms were visualized using BioMax MS film (Eastman Kodak, Rochester, NY).

For silver stain analysis, FLAG immunoprecipitations were carried out essentially as described above except a total of 2 mg of cytosolic protein was applied to the anti-FLAG resin, and adsorbed complexes were eluted off of the resin using FLAG-peptide (Sigma-Aldrich) displacement buffer (MENG, 20 mM Na₂MoO₄, and 1 mg/ml FLAG peptide). SDS-PAGE-resolved samples were stained using the Silver Stain Plus kit (Bio-Rad).

Sucrose Density Gradients. Liver cytosols were made in MENG plus 1× protease inhibitor cocktail buffer as described above and diluted in the same buffer to a protein concentration of 3.0 mg/ml as determined by BCA assay (Pierce Chemical, Rockford, IL). Cytosol samples were layered (400 μl/sample) onto 5 ml of 10 to 30% sucrose gradients. Tubes were centrifuged in a Beckman VTI 65.2 vertical rotor at 65,000 rpm for 135 min. Samples were fractionated (200 μl/fraction) using an ISCO model 640 density gradient fractionator. Fractionated proteins were then resolved by SDS-PAGE using 8% gels followed by transfer onto PVDF membrane. AHR, XAP2, and HSP90 (using anti-HSP84 and anti-HSP86 monoclonal antibodies; ABR-Affinity BioReagents) were visualized as described above. In addition, ¹²⁵I-streptavidin bands were quantified by filmless autoradiographic analysis using the Cyclone storage phosphor system in conjunction with the OptiQuant image analysis software (PerkinElmer Life and Analytical Sciences, Boston, MA).

AHR Immunoprecipitations in Hepa-1 Cells versus Mouse Liver. Hepa-1 cells were maintained at 37°C, 5% CO₂ in modified α-minimal essential media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1000 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich). Hepa-1 cytosol was prepared by homogenizing one confluent 100-mm dish of cells in 1.2 ml of buffer composed of MENG, 20 mM Na₂MoO₄, 0.1% NP-40, and 1× protease inhibitor cocktail using a steel Dounce homogenizer. Mouse liver cytosol was prepared in the same way as described above, except that the buffer used here was the same as was used for making Hepa-1 cytosol. Homogenates were centrifuged at 100,000g for 45 min to isolate cytosol. Immunoprecipitations were performed by incubating 100 μg of cytosol plus 600 μl of homogenization buffer supplemented with 2 mg/ml bovine serum albumin with 40 μl of anti-rabbit IgG agarose prebound to either nonspecific IgG or anti-AHR polyclonal IgG. Samples were mixed at 4°C for 3 h followed by four washes with homogenization buffer supplemented with 50 mM NaCl. Samples were resolved by SDS-PAGE and transferred onto PVDF membrane. Immunoblotting and filmless autoradiographic analysis was performed as described above.

Liver Cytoplasmic and Nuclear Extract Preparation. Liver (100 mg) was homogenized in 2 ml of MENG plus protease inhibitor cocktail using a glass Dounce homogenizer (20 strokes). The homogenate was spun at 1000g for 10 min, after which cytoplasmic extracts were isolated by centrifugation of the supernatant at 100,000g for 45 min. The 1000g pellet was washed one time with 2 ml of homogenization buffer plus 0.1% NP-40 followed by a 10-min 1000g spin. Nuclear extracts were then isolated by resuspending the 1000g pellet in 2 volumes of pellet wash buffer plus 400 mM KCl. The resuspended pellets were periodically vortexed over 10 min followed by

centrifugation at 21,000g for 30 min. Supernatants from the 21,000g spin were collected as the nuclear extract. Protein concentration was determined by BCA assay, and samples were resolved by SDS-PAGE. RXRα (antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a nuclear marker protein.

Microarray Analysis. Total RNA was isolated from wild-type (*n* = 3) and Tg18^{+/+} (*n* = 3) mouse livers by TRI Reagent, as described above. RNA was further purified with RNeasy kit (QIAGEN), according to the manufacturer's protocol. The quality of RNA was confirmed by agarose gel electrophoresis and on Bioanalyzer (Agilent Technologies, Palo Alto, CA). Thereafter, total RNA was labeled using One-Cycle Target Labeling and Control reagents (Affymetrix, Santa Clara, CA) and scanned with GeneChip Scanner 3000 at the microarray core facility of The Huck Institutes of Life Sciences (The Pennsylvania State University). Microarray experiments were performed using the GeneChip Mouse Genome 430 2.0 arrays comparing transgenic versus wild-type mice (4-week-old females).

Raw data files were subjected to background adjustment, normalization, and summarization using RMAexpress (0.3 release; Irizarry et al., 2003). Thereafter, the output from RMAexpress was analyzed by Significance Analysis of Microarrays (SAM, version 2.23A; Tusher et al., 2001) with the following selections: 100 permutations, K-nearest neighbors' imputer, and 10 neighbors. Expression value comparisons were deemed significant at a δ value of 1.0 and 2-fold change. Thirty-six probe-sets were found to be significant with a false discovery rate of 2% (Tables 1 and 2). Genes found to be significantly under-expressed in XAP2 transgenic mice were further analyzed by QRT-PCR.

Results

Transgenic Mice Display Enhanced Expression of Hepatic XAP2 That Exists in a Complex with the AHR.

In the liver, the AHR serves to regulate the expression of numerous enzymes involved in endobiotic and xenobiotic metabolism. XAP2 shows considerable variability in protein levels present between tissues, and in liver it is relatively low compared with some nonhepatic tissues (Fig. 1A). This raises the question of whether XAP2 is a limiting component in modulating AHR activity in the liver in comparison with some higher expressing tissues, such as spleen, thymus, and brain. To address this question, transgenic mouse lines were created in the C57BL/6J strain background that resulted in elevated XAP2 expression under the transcriptional control of the mouse transthyretin promoter. Mouse XAP2 cDNA, containing a carboxyl-terminal FLAG-tag, was cloned into exon 2 of the TTR1exV3 vector (Fig. 1B), which has been used by numerous laboratories, resulting in transgenic mouse lines that exhibit robust liver-specific expression of exogenous genes (Wu et al., 1996; Mallet et al., 2002). Mice that contained the XAP2-FLAG transgene were identified by a PCR-based screen (Fig. 1C) and two founder lines, designated Tg04 and Tg18 were used for subsequent studies.

To confirm that XAP2-FLAG expression was isolated to the liver in the transgenic mouse lines, whole cell extracts were prepared using tissues collected from wild-type, Tg04^{+/+}, and Tg18^{+/+} animals. In both transgenic lines, but not in wild type, XAP2-FLAG expression seemed to be present only in the liver of these animals (Fig. 2A). In cell culture and other in vitro systems XAP2-FLAG is capable of existing in AHR complexes to the same degree as the normal nontagged form (Meyer et al., 2000). To validate that it does exist in the unliganded AHR complex in the transgenic mouse lines, FLAG immunoprecipitations were performed using liver cy-

tosols from wild-type, Tg04^{+/-}, and Tg18^{+/-} mice. Immunoblot results (Fig. 2B) confirmed that XAP2-FLAG was present in a complex with the AHR and HSP90.

Wild-Type and Transgenic Mice Have Similar Gene Expression Patterns Both Basally and after AHR Ligand Activation. Increased XAP2 expression through exogenous means has been demonstrated to modulate AHR transcriptional activity in cell culture and yeast systems (Carver et al., 1998; LaPres et al., 2000; Hollingshead et al., 2004). The ability of XAP2 to alter AHR transcriptional properties in animal systems, however, has yet to be addressed. To that end, male wild-type and transgenic homozygous mice were administered the moderate affinity AHR ligand β -NF via intraperitoneal injection for 6 h using a broad range of doses (from 1×10^{-3} to 2.5×10^2 mg/kg). We previously reported that XAP2-mediated repression of AHR activity is most pronounced using subsaturating doses of the moderate affinity AHR ligand iodoflavone (Petrulis et al., 2003). Therefore, β -NF was chosen as the ligand for these experiments based

on its similarity to iodoflavone, both in structure and AHR-binding affinity. The dose-response curve generated for CYP1A1 mRNA shows no difference in expression levels in wild-type versus both the Tg04^{+/+} and Tg18^{+/+} mouse lines (Fig. 3A). The dose-response relationship of CYP1A1 mRNA levels agrees nicely with the previously reported studies in C57BL/6J mice documenting aryl hydrocarbon hydroxylase activity in response to β -NF treatment (Niwa et al., 1975). In addition, it is noteworthy that no differences in CYP1A1 expression were observed between genotypes in females as well (data not shown). The expression levels of other AHR responsive genes were also measured. No difference in the expression of CYP1A2, UGT1A2, NQO1, CAR, or NRF2 was observed in control samples or samples collected from mice exposed to subsaturating concentrations (20 mg/kg) of β -NF (Fig. 3, B–F). In response to a saturating concentration (250 mg/kg) of β -NF, statistically different levels of CYP1A2 (reduced) and NQO1 (increased) mRNA were detected. However, it seems unlikely that these modest changes in mRNA

TABLE 1

Genes that showed increased expression in transgenic versus wild-type mice

Affymetrix Probe ID	Gene Symbol	Gene Title	-Fold Change
1423128_at	<i>Aip</i>	Aryl-hydrocarbon receptor-interacting protein	39.5
1448194_a_at	<i>H19</i>	H19 fetal liver mRNA	13.5
1438617_at			6.2
1451612_at			3.4
1433837_at	<i>8430408G22Rik</i>	RIKEN cDNA 8430408G22 gene	3.1
1448510_at	<i>Efnal</i>	Ephrin A1	2.9
1416895_at	<i>Efnal</i>	Ephrin A1	2.8
1435872_at			2.7
1438519_at	<i>4930429H24Rik</i>	RIKEN cDNA 4930429H24 gene	2.6
1431240_at	<i>Clrf</i>	C-type lectin-related f	2.5
1439331_at	<i>4932439E07Rik</i>	RIKEN cDNA 4932439E07 gene	2.4
1453145_at	<i>4933439C20Rik</i>	RIKEN cDNA 4933439C20 gene	2.2
1433836_a_at	<i>8430408G22Rik</i>	RIKEN cDNA 8430408G22 gene	2.2
1420699_at	<i>Clecsf12</i>	C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 12	2.2
1429900_at	<i>5330406M23Rik</i>	RIKEN cDNA 5330406M23 gene	2.2
1435458_at	<i>Pim1</i>	Proviral integration site 1	2.1
1426208_x_at	<i>Plagl1</i>	Pleiomorphic adenoma gene-like 1	2.1
1440311_at	<i>Sorbs1</i>	Sorbin and SH3 domain containing 1	2.0

TABLE 2

Genes that showed decreased expression in transgenic versus wild-type mice

Affymetrix Probe ID	Gene Symbol	Gene Title	-Fold Change
1425837_a_at	<i>Cern4l</i>	CCR4 carbon catabolite repression 4-like (<i>Saccharomyces cerevisiae</i>)	5.3*
1421669_at	<i>Sult3a1</i>	Sulfotransferase family 3A, member 1	4.3*
1428223_at	<i>1700018O18Rik</i>	RIKEN cDNA 1700018O18 gene	3.9*
1419136_at	<i>Akr1c18</i>	Aldo-keto reductase family 1, member C18	3.4*
1419704_at	<i>Cyp3a41</i>	Cytochrome P450, family 3, subfamily a, polypeptide 41	2.7*
1418028_at	<i>Dct</i>	Dopachrome tautomerase	2.7*
1440921_at			2.6
1418025_at	<i>Bhlhb2</i>	Basic helix-loop-helix domain containing, class B2	2.4*
1422257_s_at	<i>Cyp2b20</i> /// <i>Cyp2b10</i>	Cytochrome P450, family 2, subfamily b, polypeptide 20 /// cytochrome P450, family 2, subfamily b, polypeptide 10	2.4
1425645_s_at	<i>Cyp2b20</i> /// <i>Cyp2b10</i>	Cytochrome P450, family 2, subfamily b, polypeptide 20 /// cytochrome P450, family 2, subfamily b, polypeptide 10	2.3
1445641_at			2.3
1429169_at	<i>Rbm3</i>	RNA-binding motif protein 3	2.2
1451787_at	<i>Cyp2b20</i>	Cytochrome P450, family 2, subfamily b, polypeptide 20	2.2
1434449_at	<i>Aqp4</i>	Aquaporin 4	2.2
1435595_at	<i>1810011O10Rik</i>	RIKEN cDNA 1810011O10 gene	2.1
1438035_at	<i>AW061290</i>	Expressed sequence AW061290	2.1
1420772_a_at	<i>Dsrip1</i>	Delta sleep-inducing peptide, immunoreactor	2.0*
1415834_at	<i>Dusp6</i>	Dual-specificity phosphatase 6	2.0*

* Genes that were confirmed by QRT-PCR to be different between genotypes.

would result in a biologically relevant modulation in CYP1A2 or NQO1 protein levels. It should be noted that we have recently identified CAR to be inducible through an AHR-dependent mechanism (R. D. Patel, B. D. Hollingshead, and G. H. Perdew, manuscript submitted for publication). Because expression of the genes UGT1A2, NQO1, and NRF2 could be induced indirectly by oxidant stress caused by β -NF metabolism, we wanted to be sure that their induction was AHR-dependent. As seen in Fig. 3G, AHR^{-/-} mice showed no increase in UGT1A2, NQO1, or NRF2 showing that their induction by β -NF was through activation of the AHR. As expected CYP1A1 expression was virtually undetectable in AHR^{-/-} mice. Wild-type mice treated in parallel with AHR^{-/-} mice displayed β -NF-induced increases in expression of CYP1A1, UGT1A2, NQO1, and NRF2 (data not shown).

PPAR α Transcriptional Activity Is Unaltered in Wild-Type versus Transgenic Mice. XAP2 also has been shown to attenuate the transcriptional activity of the nuclear receptor PPAR α in cell culture systems (Sumanasekera et al., 2003). To see whether hepatic PPAR α activity was altered in the presence of increased XAP2 levels wild-type and Tg04^{+/+} mice were administered the PPAR α -specific ligand Wy-14,643 via gavage. The mRNA levels of two PPAR α responsive genes, ACOX1 and FABPL, were

efficiently induced in Wy-14,643 treated mice. However, no differences in mRNA amounts were observed between wild-type and Tg04^{+/+} mice in control or ligand-induced samples (Fig. 4). It seems as though increased XAP2 expression in the liver exerts no effects on PPAR α transcriptional activity.

Stoichiometric Binding of XAP2 in the AHR Complex Is Similar in Wild-Type and Transgenic Mice. Because AHR transcriptional capacity seems to be unaltered by the enhanced expression of XAP2, we wanted to see whether XAP2 occupancy in the cytosolic AHR complex was changed. First, sucrose density fractionation of wild-type and Tg18^{+/+} liver cytosol was performed, and samples were resolved by SDS-PAGE. Immunoblot detection of AHR, HSP90, and XAP2 was performed using ¹²⁵I-streptavidin, and bands were quantified by filmless autoradiographic analysis. Although Tg18^{+/+} samples show a broader XAP2 elution profile than wild-type samples, enrichment of XAP2 in the AHR-positive fractions is not observed (Fig. 5A). Most cellular XAP2 seems to exist in small complexes or as a monomer. This implies that XAP2 presence in the AHR complex may be the same between wild-type and transgenic animals. To distinguish with certainty if this is indeed correct AHR immunoprecipitations were performed, and the amount of XAP2 coprecipitated with the receptor was assessed. As suspected, little

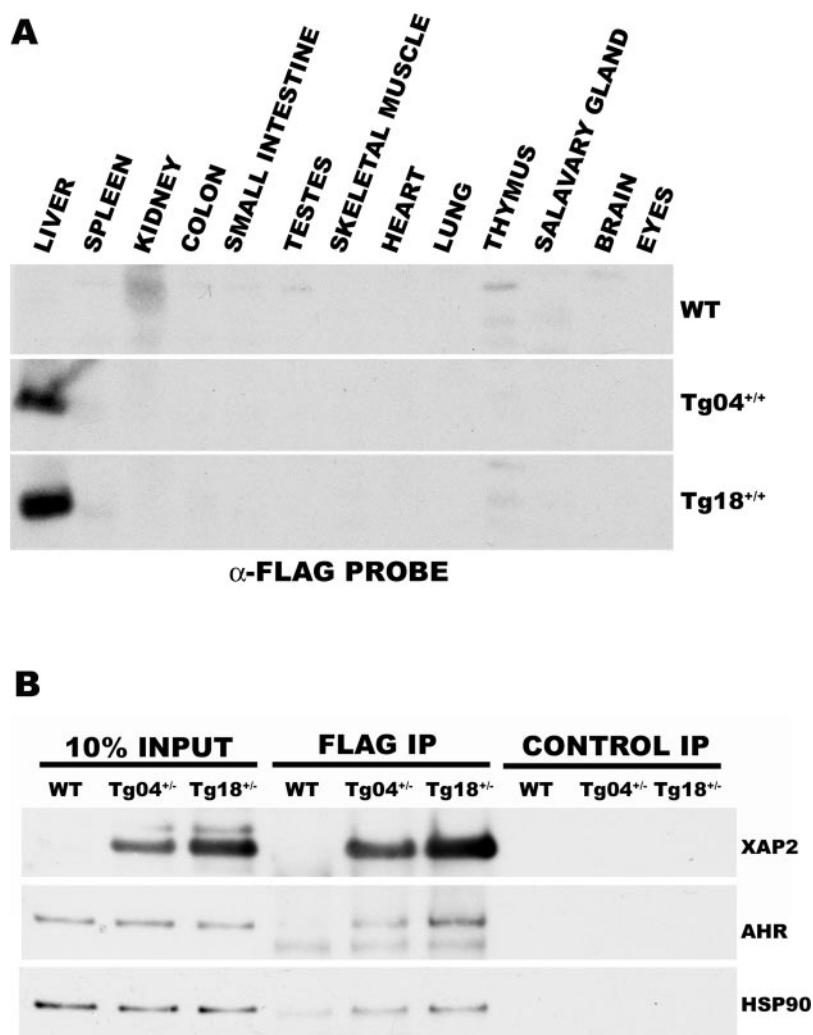


Fig. 2. Transgenic XAP2 expression is localized to the liver and is found in a complex with the AHR. A, a variety of tissues were isolated, and cytosol was prepared from male wild-type, Tg04^{+/+}, and Tg18^{+/+} mice. The expression of the XAP2-FLAG transgenic protein was assessed by immunoblotting using an anti-FLAG antibody. No expression of XAP2-FLAG could be found in any of the nonhepatic tissues examined. B, immunoprecipitations of XAP2-FLAG were performed using liver cytosol made from wild-type, Tg04^{+/+}, and Tg18^{+/+} mice. Immunoblotting confirmed that XAP2-FLAG is capable of existing in a complex with the AHR and HSP90.

difference in coprecipitated XAP2 was observed in wild-type versus hemi- and homozygous transgenic animals (Fig. 5B), even given the large increase in XAP2 expression in the transgenic lines (Fig. 5B, inputs). Filmless autoradiographic analysis quantification of XAP2 coadsorbed with the AHR

demonstrated that no increase (hemizygous) or less than a 2-fold increase (homozygous) of XAP2 was present in transgenic AHR complexes (data not shown). Taken together, these results would lead to the conclusion that normal liver expression of XAP2, although low relative to some nonhe-

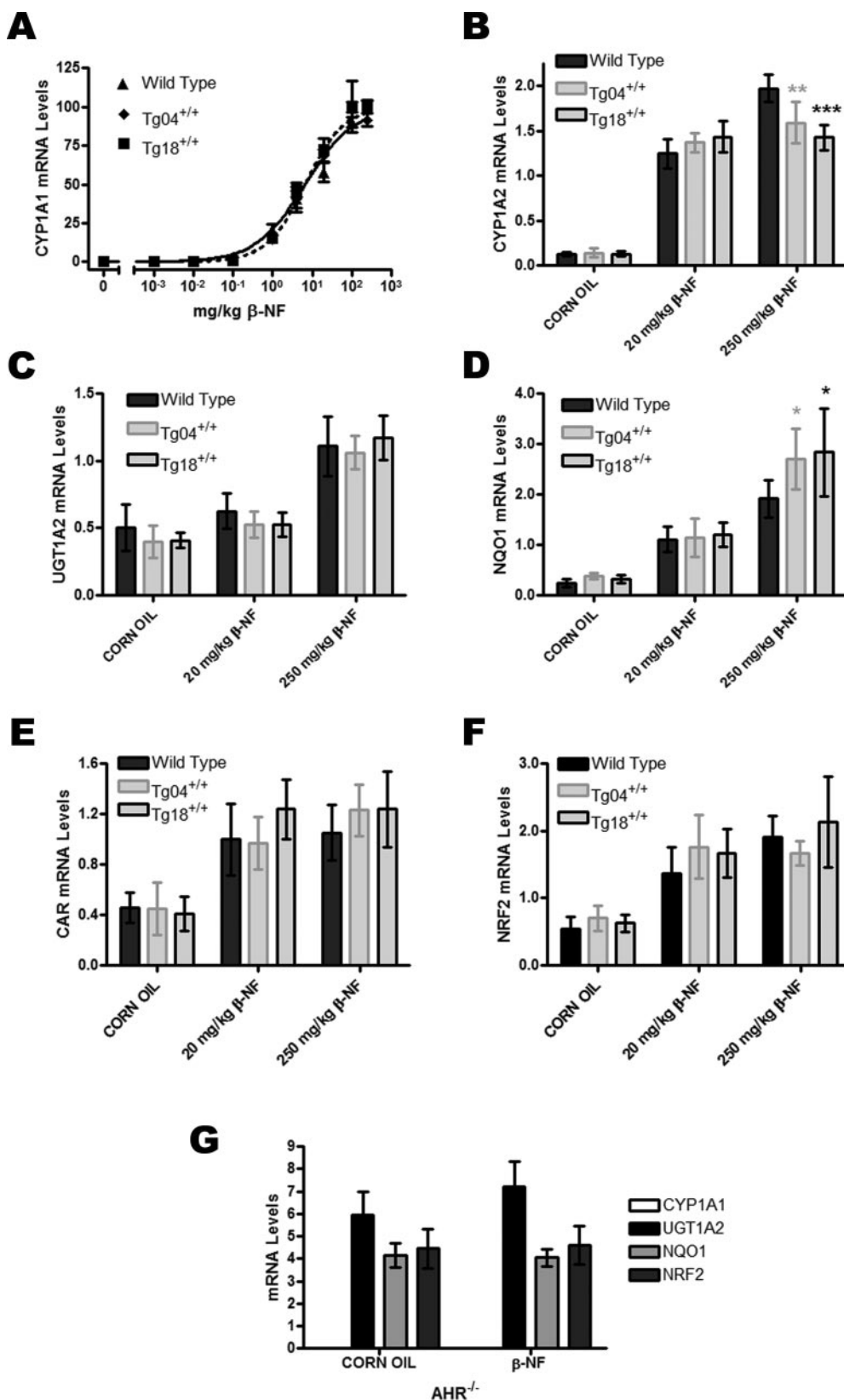


Fig. 3. AHR regulation of target genes is unaltered in wild-type versus transgenic mice upon exposure to nonsaturating and saturating concentrations of an AHR ligand. Male mice between 6 and 10 weeks of age were injected intraperitoneally with various concentrations of β -NF (10^{-3} to 2.5×10^2 mg/kg) or corn oil vehicle for 6 h. QRT-PCR was used to quantify mRNA expression of selected genes. A, a full dose-response curve of CYP1A1 expression was generated, observing no change in CYP1A1 mRNA content between genotypes at any β -NF dose. The expression levels of CYP1A2 (B), UGT1A2 (C), NQO1 (D), CAR (E), and NRF2 (F) mRNA were also unchanged between genotypes in control and β -NF-treated (20- and 250-mg/kg) samples. The number of animals used for each treatment group ($n = 8$) except β -NF concentrations of 1.0 and 2.5×10^2 mg/kg ($n = 4$). (G) AHR^{-/-} mice ($n = 3$) were injected intraperitoneally with 50 mg/kg β -NF or corn oil for 5 h. All values shown are normalized relative to GAPDH levels. Data presented are mean values, and error bars represent standard error (A) or standard deviation (B–G) of those samples. (*, **, ***, $p \leq 0.05$, 0.01, or 0.001, respectively).

patic tissues, is sufficient to obtain maximal occupancy in AHR complexes.

AHR Complexes in Hepa-1 Cells and Mouse Liver Are Saturated with XAP2. Contrary to a previous report that concluded that, in Hepa-1 cells, XAP2 is present in ~40% of AHR complexes (Petrulis et al., 2000), a recent study using these cells determined that AHR complexes are completely saturated with XAP2 (Pollenz et al., 2006). Because hepatic AHR complexes in mouse liver also seem to be maximally associated with XAP2, we wanted to assess the relative levels of AHR and XAP2 between the two. As seen in Fig. 6A, the levels of the AHR (3-fold) and XAP2 (4-fold) are elevated in Hepa-1 cells compared with mouse liver. Immunoprecipitation of the AHR from Hepa-1 and liver cytosols results in an equal proportion of XAP2 coadsorption relative to the amount of AHR precipitated. The results from this experiment further support that the AHR in Hepa-1 and mouse liver cytosols is maximally bound with XAP2.

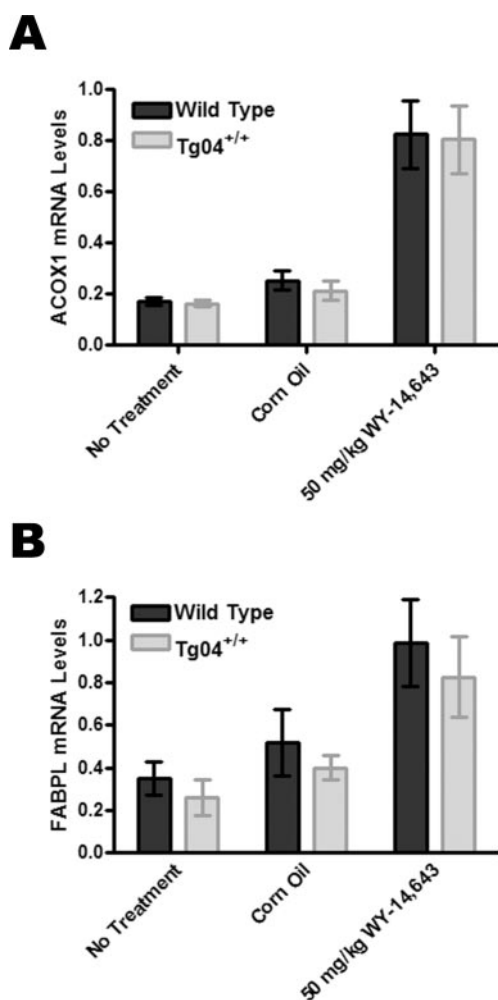


Fig. 4. PPAR α -regulated gene expression is unaltered between wild-type and transgenic mice in the presence and absence of Wy-14,643 treatment. Wild-type or Tg04^{+/+} males were left untreated ($n = 3$) or were administered the PPAR α -specific ligand Wy-14,643 ($n = 6$) or corn oil vehicle ($n = 3$) via gavage and livers removed after 7 h. QRT-PCR was used to quantify mRNA levels of ACO1 (A) and FABPL (B). No statistical difference was observed between mRNA levels in wild-type versus Tg04^{+/+} mice. All values shown are normalized relative to GAPDH levels. Data presented are mean values, and error bars represent standard deviation of those samples.

AHR in Wild-Type and Transgenic Livers Is Predominantly Localized in the Cytoplasm. XAP2 is known to cause cytoplasmic sequestration of the AHR in cell culture systems (Petrulis et al., 2000). To determine whether AHR cellular localization is altered in transgenic compared with wild-type mice, cytoplasmic and nuclear extracts were prepared from wild-type and Tg04^{+/+} mouse livers. Immunoblot analysis reveals that in wild-type mice, AHR is localized almost exclusively in the cytoplasm with only a minor fraction being found in the nucleus (Fig. 6B). RXR α was used as a positive control for constitutively nuclear localization. In transgenic samples the AHR shows identical cellular distribution as found in wild-type samples. In addition, XAP2 is found to be predominantly cytoplasmic in wild-type and transgenic liver samples with some nuclear localization.

Transgenic XAP2 Binds to HSP90 in High Abundance. To determine whether XAP2 binds to any previously unidentified cytosolic proteins transgenic XAP2 was immunoprecipitated with anti-FLAG resin. Adsorbed XAP2-FLAG complexes were eluted off the resin, and TSDS-PAGE resolved samples were visualized by silver stain procedure. Two approximately 90-kDa proteins were found to coadsorb with XAP2-FLAG, and they are likely isoforms of HSP90 (Fig. 7). AHR coimmunoprecipitation cannot be determined because of the nonspecific 97-kDa band that is found to migrate at the same molecular mass as the AHR. No other high abundance interactions with XAP2 were observed implying that XAP2 may exist largely as a monomer or bound to HSP90, which is in agreement with the sucrose density gradient studies (Fig. 5A).

The Identification of Genes Whose Expression Is Altered by Elevated XAP2 Levels. Gene expression studies were performed using the Affymetrix GeneChip Mouse Genome 430 2.0 arrays in an attempt to identify previously uncharacterized XAP2-regulated genes. Probe-sets that displayed a 2-fold or greater difference in expression (see *Materials and Methods* section for details of data analysis) between genotypes are shown in Tables 1 and 2. As expected, XAP2 shows the largest difference in expression between wild-type and Tg18^{+/+} samples. Most of the probe-sets that show elevated expression (Table 1) in transgenic compared with wild-type samples do not represent well defined genes making it difficult to speculate how XAP2 might serve to modulate their function. It is noteworthy that mRNA expression of the phase I CYP3A41, and CYP2B20 enzymes and the phase II enzyme SULT3A1 are repressed in transgenic samples (Table 2). In addition, a number of other genes encoding functionally dissimilar enzymes are repressed in transgenic mice livers. Differential mRNA expression for a subset of array targets was verified using QRT-PCR, and genes that were confirmed to be dissimilar between genotypes are indicated in Table 2 by an asterisk. In the future it will be of interest to see which of the genes are indeed genuinely regulated by XAP2 and how their function can be altered through XAP2-mediated modulation.

Discussion

As mentioned previously, the AHR regulates the expression of a number of genes involved in the metabolic clearance of exogenous compounds. This seemingly benign biological role the AHR plays in metabolism is in opposition to the toxic

endpoints, which occur in response to prolonged AHR transcriptional activity, such as hepatomegaly (Fernandez-Salguero et al., 1996) and hepatocarcinogenesis (Moennikes et al., 2004). Because of the delicate interplay between physiological and toxic endpoints mediated through AHR activity, it is necessary to obtain a better mechanistic understanding of AHR regulation. Previous studies have shown that AHR transcriptional control is modulated by the AHR oligomeric complex chaperone proteins HSP90 and XAP2. Because overall XAP2 levels are highly variable between tissues relative to HSP90 (Fig. 1A), it was considered that differential expression of XAP2 may perhaps serve as a mechanism to regulate AHR activity between tissues. If indeed XAP2 does modulate AHR differently between tissues, then it is possible that the XAP2 levels within a tissue could enhance or atten-

uate the susceptibility of individual tissues to AHR-mediated toxicity.

The creation of transgenic mouse lines under the transcriptional control of the mouse transthyretin promoter resulted in founder lines that contained liver-specific XAP2 expression in levels greater than any other tissue examined (data not shown). Given this large increase in hepatic XAP2, it was quite surprising that AHR transcriptional output was observed to be virtually identical between wild-type and transgenic mouse lines (Fig. 3). We previously noted that XAP2-mediated repression of AHR activity was most pronounced at subsaturating levels of AHR ligand (Petrulis et al., 2003). Therefore, the mRNA levels for numerous metabolic (CYP1A1, CYP1A2, NQO1, and UGT1A2) or metabolically relevant (CAR and NRF2) genes were examined at saturat-

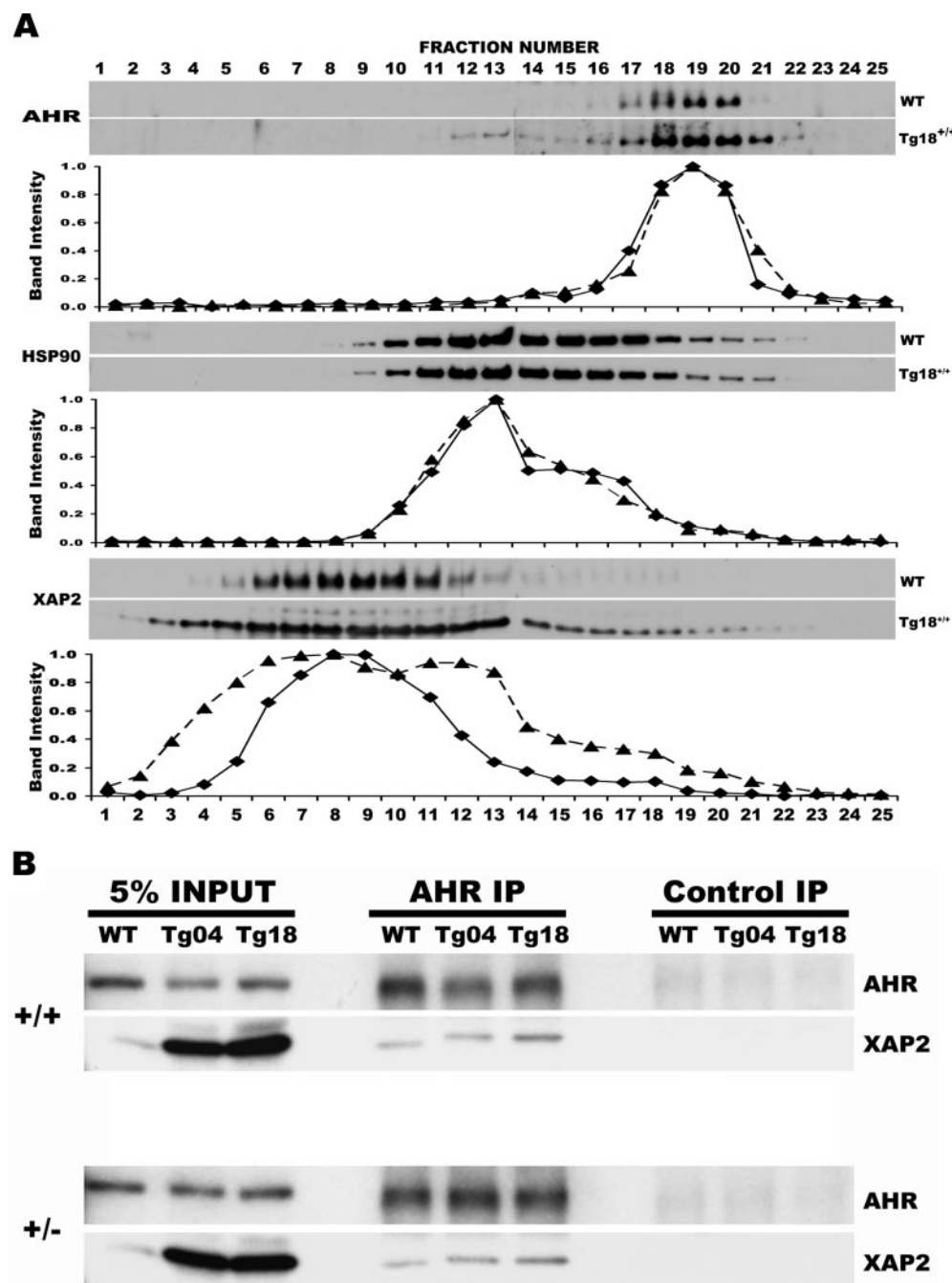


Fig. 5. The presence of XAP2 in AHR complexes is stoichiometrically similar between wild-type and transgenic mice. **A**, liver cytosol samples (1.2 mg per sample) from wild-type and Tg18^{+/+} mice were fractionated on 10–30% sucrose gradients. Aliquots from each fraction were resolved by TSDS-PAGE and visualized by immunoblot using biotin-conjugated secondary antibodies in combination with ¹²⁵I-streptavidin. Respective protein bands were quantified by filmless autoradiographic analysis. Diamonds and triangles represent data values obtained for wild-type and Tg18^{+/+} samples, respectively. **B**, AHR immunoprecipitations of liver cytosol samples from wild-type and transgenic (hemizygous and homozygous) mice were performed. TSDS-PAGE-resolved samples were visualized by immunoblot using biotin-conjugated secondary antibodies in combination with ¹²⁵I-streptavidin.

ing (100 and 250 mg/kg) and subsaturating (<100 mg/kg) amounts of the AHR ligand β -NF. At all doses examined, gene expression was virtually unchanged between genotypes. NRF2 has recently been demonstrated to be an AHR-inducible gene (for review, see Kohle and Bock, 2006). Because NRF2 also regulates the transcription of numerous phase II enzymes, it is possible that the elevated expression of NQO1 and UGT1A2 observed here after β -NF treatment is due to the combinatorial regulation of these genes by the AHR and NRF2. In addition, transcriptional activity of PPAR α was unaltered between genotypes (Fig. 4) unlike what has been observed in cell culture (Sumanasekera et al., 2003). Further work must be done to mechanistically explain discrepancies between the cell culture and in vivo reports of the ability of

XAP2 to attenuate PPAR α activity. Despite these negative results, the microarray data suggest that enhanced XAP2 expression is capable of influencing gene expression. It should be noted that two pathologists observed no significant morphological differences between wild-type and transgenic formalin-fixed liver sections prepared from 2-, 4-, and 6-week-old mice.

To mechanistically explain why AHR transcriptional properties were unaltered, we examined relative levels of HSP90 and XAP2 in the unliganded AHR complex. Sucrose density gradient fractionation and immunoprecipitation experiments (Figs. 5 and 6A) highlighted that most XAP2 in wild-type animals is not bound along with the AHR. Furthermore, increasing the amount of XAP2 available for binding in

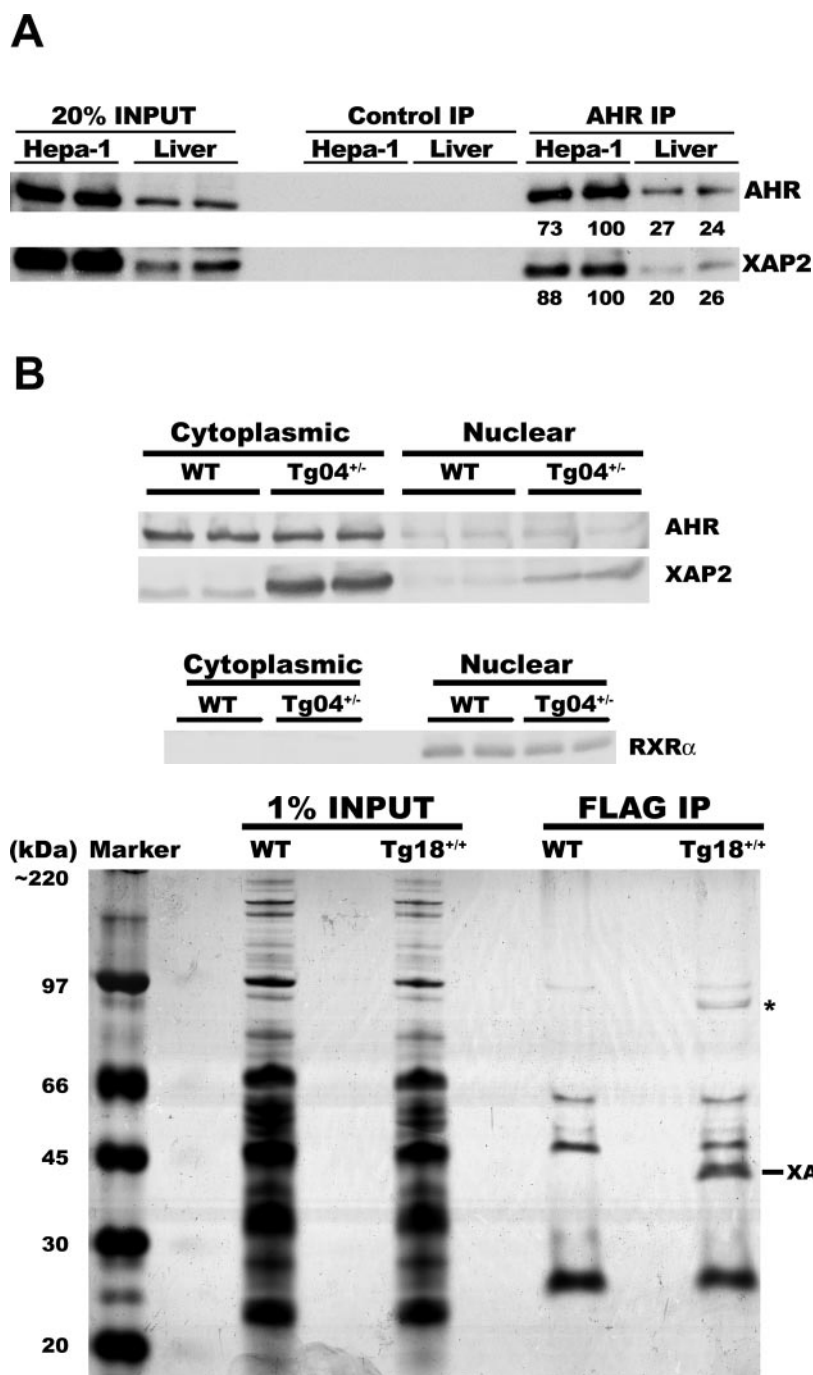


Fig. 6. The assessment of AHR-XAP2 complex stoichiometry in cell culture versus in liver, and the cellular localization of the AHR in wild-type and transgenic mice. A, AHR immunoprecipitations were performed using Hepa-1 or wild-type mouse liver cytosol isolated from two animals. TSDS-PAGE resolved samples were visualized by immunoblot using biotin-conjugated secondary antibodies in combination with ¹²⁵I-streptavidin. The intensity of radio-labeled bands corresponding to the AHR and XAP2 was quantified by filmless autoradiographic analysis. Numerical values shown beneath the bands are representative of the amount of the AHR precipitated or XAP2 coprecipitated in each sample relative to the highest intensity band. B, cytoplasmic and nuclear extracts were prepared from livers of wild-type and Tg04^{+/-} mice. Equal amounts of cytoplasmic and nuclear protein were resolved by TSDS-PAGE and samples were visualized by immunoblot using biotin-conjugated secondary antibodies in combination with ¹²⁵I-streptavidin. RXR α was used as a nuclear marker protein.

Fig. 7. Screen for novel XAP2-interacting proteins. XAP2-FLAG was immunoprecipitated from wild-type and Tg18^{+/+} liver cytosol. Adsorbed complexes were eluted off the anti-FLAG resin samples were resolved by TSDS-PAGE followed by protein visualization by silver stain. Asterisk indicates specific XAP2-interacting proteins at ~90 kDa, likely HSP90.

transgenic animals resulted in no appreciable increase in the stoichiometric binding of XAP2 in the AHR complex. This result was surprising considering that a previous stoichiometric evaluation of AHR complexes in Hepa-1 cells found that only ~40% of cytosolic AHR is bound to XAP2 (Petrulis et al., 2000). However, a recent report has questioned this finding suggesting that, in Hepa-1 cells, AHR complexes are endogenously saturated with XAP2 (Pollenz et al., 2006). The results presented in Fig. 6A show that the AHR in Hepa-1 cells and wild-type liver cytosol binds to XAP2 with the same stoichiometry. This finding provides strong support for the idea that the cytosolic hepatic AHR is maximally complexed with the AHR. In addition, experiments performed using in vitro-translated AHR and XAP2 demonstrated that XAP2 has a high affinity for the AHR and that complete association of XAP2 with the AHR occurred after the mixing together of individually translated components (Meyer et al., 1998). XAP2 expression is lower in liver than in many other tissues (Fig. 1A), yet XAP2 seems to be present in excess molar quantities compared with the AHR. Therefore, XAP2 in all probability does not regulate AHR function in various tissues by means of its differential expression. Taken together, it would seem as though that the endogenous levels of XAP2 in mouse liver are more than sufficient to allow for maximal formation of AHR-HSP90-XAP2 complexes.

Previous cell culture studies have demonstrated the ability of XAP2 to stabilize the AHR, resulting in increased cytosolic levels and elevated AHR transcriptional activity (Kazlauskas et al., 2000; LaPres et al., 2000; Lees et al., 2003). AHR complexes isolated from Hepa-1 cells and wild-type mouse liver are each maximally associated with XAP2. Unlike Hepa-1 cells, however, the increased expression of XAP2 in vivo does not result in the elevation of total cytosolic AHR levels. AHR levels in transgenic animals were consistently found to be identical to wild-type mice (Fig. 2B, 5B, and 6B), and therefore it seems as though the stabilization of the AHR and enhanced transcriptional activity that occurs in cell culture after increased XAP2 expression is an artifact of the experimental system used. This conclusion is supported by a recent report that showed only a modest decrease in AHR levels after the knock-down of XAP2 expression in Hepa-1 cells (Pollenz et al., 2006). It is also possible that because hepatocytes in vivo are fundamentally different than cells grown on plastic culture dishes that some other factors regulate AHR function in vivo that have yet to be discovered.

XAP2-mediated repression of AHR activity in cell culture has been speculated to result from its ability to sequester the AHR in the cytoplasm and displace p23 from the AHR complex (Hollingshead et al., 2004). In vivo, however, the AHR seems to normally be exclusively cytoplasmic in wild-type animals with no change in cellular localization in transgenic lines. Incorporation of p23 in the AHR complex in vivo could not be detected (data not shown). It is likely that because the AHR is maximally bound to XAP2 in vivo, XAP2 is responsible for the cytoplasmic localization of the AHR. If this is indeed true then XAP2 may play a critical role in regulating AHR function that cannot be addressed using the transgenic model presented in this study. The creation of XAP2 null mice will address whether this regulatory mechanism occurs in vivo.

Epidemiological studies in human populations have shown interindividual variability in AHR ligand-binding affinity

and CYP1 inducibility that cannot be explained by AHR polymorphisms (Nebert et al., 2004). Therefore, it is possible that differential expression of chaperone proteins such as HSP90, XAP2, and p23 could play a role in some of these observed variabilities. Therefore, the information collected in this study is useful in that it seems to suggest that interindividual differences in AHR activity are not mediated through varying XAP2 levels. However, the total absence of XAP2 expression may influence AHR activity.

Experiments carried out to determine whether XAP2 binds to any previously unidentified cytosolic proteins identified no new protein interactions (Fig. 7A). These results by no means conclude that XAP2 does not bind other unidentified proteins, because other non-AHR complex interactions have already been characterized, but it suggests that additional high abundance interactions do not exist among soluble proteins in the liver. We also provide a list of genes that were differentially regulated in wild-type and transgenic animals. Future studies will determine whether any of these genes are regulated by the endogenous XAP2 normally found at low levels in mouse liver, or whether their altered expression is simply due to the enhanced expression of XAP2 in these transgenic lines.

It is evident from this report that hepatic XAP2 is not limiting within the context of AHR complexes as hypothesized. Future work using XAP2 null mouse lines needs to be performed to determine what kind of role the endogenous pool of XAP2 plays in modulating AHR function. It will be of interest to note whether XAP2 is saturating in its ability to regulate hepatic AHR activity, as inferred from this work, or whether XAP2 is completely nonessential in the context of regulating the hepatic AHR. In addition, whether XAP2 modulates nonhepatic development of AHR-mediated phenotypes, such as the endothelial cell-mediated vascular defect in the fetal liver (Walisser et al., 2005) may be of interest to explore in the future.

Acknowledgments

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