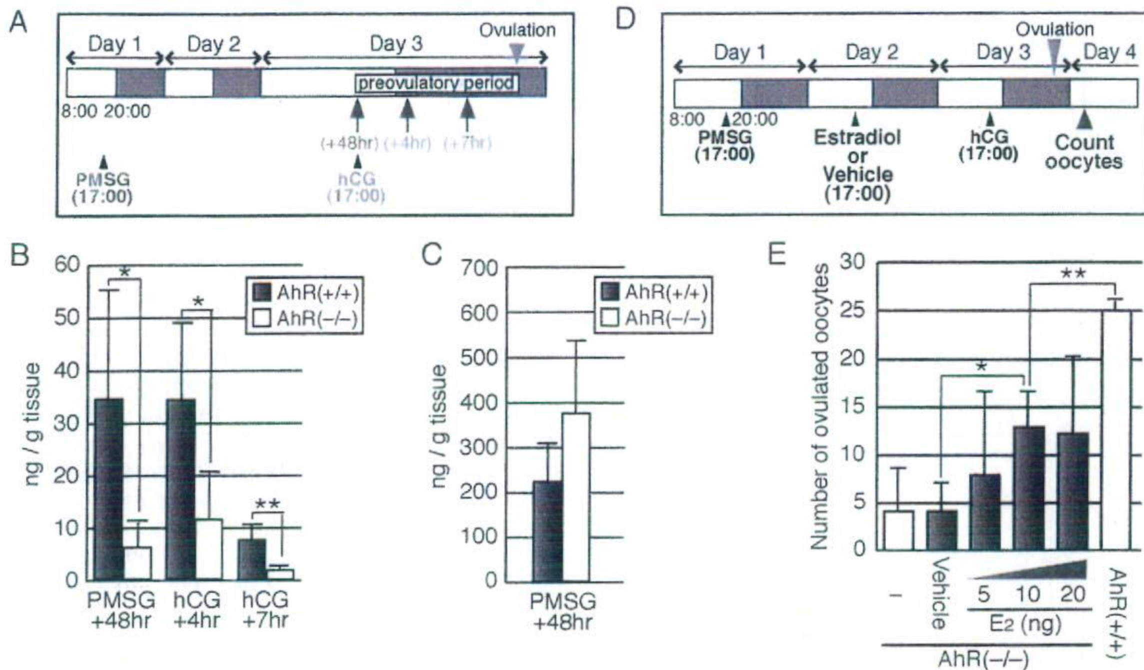


FIG. 3. Concentrations of intraovarian steroids in AhR$^{-/-}$ females and the rescue of ovulation by estradiol treatment. (A) Schematic representation of the experimental procedure used to determine intraovarian steroid concentrations during the preovulatory period. (B) Intraovarian estradiol concentrations in AhR$^{+/+}$ and AhR$^{-/-}$ females. The ovaries of at least three AhR$^{+/+}$ and three AhR$^{-/-}$ female mice were collected at the times indicated in panel A. Estradiol concentrations were then determined by liquid chromatography-mass spectrometry analysis. *, $P < 0.10$; **, $P < 0.05$. (C) Intraovarian testosterone concentrations were determined as described for panel B. (D) Schematic representation of the experimental estradiol administration procedure used to rescue the ovulation of AhR$^{-/-}$ mice. Mice treated with PMSG at day 1 were divided into two groups. One group was treated with various quantities of estradiol, while the other group was given vehicle alone on day 2. The mice from both groups were treated with hCG on day 3, and ovulation was assessed at day 4. (E) Effects of estradiol administration on ovulation in AhR$^{-/-}$ females. After the treatment of AhR$^{+/+}$ and AhR$^{-/-}$ females with PMSG and hCG, the oocytes released by ovulation were counted (open bars). AhR$^{-/-}$ females were also given an intraperitoneal injection of 5 to 20 ng 17β-estradiol (E2) or vehicle alone (filled bars) as described for panel D prior to counting the ovulated oocytes. *, $P < 0.025$; **, $P < 0.005$.

tochemistry that Cyp19 protein levels were diminished in the granulosa cells of AhR$^{-/-}$ ovaries (Fig. 4F).

AhR directly activates *Cyp19* gene transcription in cooperation with an orphan nuclear receptor, Ad4BP/SF-1. As the previously described results strongly suggest the involvement of AhR in *Cyp19* expression, we examined the mechanism by which AhR regulated *Cyp19* gene transcription. The *Cyp19* gene has multiple tissue-specific first exons (23, 33, 55). In the ovary, this gene is transcribed from exon PII (Ex 1d) in a mechanism involving the orphan nuclear receptor Ad4BP/SF-1 (8, 32, 40, 45). The binding site for Ad4BP/SF-1 is conserved within the 5′ upstream regions of the human and mouse genes. We also determined that the human *CYP19* and mouse *Cyp19* genes have an AhR/Arnt-binding sequence (XRE) 3,756 and 5,058 bp upstream of the ovary-specific first exon, respectively (Fig. 5A and B). We therefore transiently transfected the expression vectors of AhR, Arnt, and Ad4BP/SF-1 into cultured cells to investigate the promoter function of the *CYP19/Cyp19* genes. While Ad4BP/SF-1 clearly activated *CYP19/Cyp19* gene transcription, cotransfection of AhR and Arnt resulted in only weak activation. Simultaneous expression of AhR/Arnt with Ad4BP/SF-1, however, synergistically activated the Cyp19 promoter (Fig. 5C and D). Subsequent expression of AhRR suppressed the transcription activation induced by AhR (Fig. 5C and D).

The observed synergistic activation of the *CYP19/Cyp19* promoter by AhR/Arnt and Ad4BP/SF-1 implied a physical interaction between these proteins. To verify this interaction, we cotransfected expression vectors encoding 3×FLAG-AhR, Arnt, and EGFP-Ad4BP/SF-1 and then attempted to coimmunoprecipitate these components by using an anti-FLAG antibody. EGFP-Ad4BP/SF-1, but not EGFP, was coimmunoprecipitated with FLAG-AhR (Fig. 6A), indicating a potential physical interaction between AhR/Arnt and Ad4BP/SF-1. To investigate if AhR binds to the XRE within the promoter of *Cyp19* in vivo, we performed a ChIP assay using chromatin isolated from the granulosa cells of gonadotropin-treated ovaries (Fig. 6B). PCR analysis of the immunoprecipitates isolated using an anti-AhR antibody (Fig. 6B) revealed that the XRE of the *Cyp19* gene was associated with AhR in samples derived from wild-type mice but not AhR$^{-/-}$ mice (Fig. 6C). This result clearly indicates that AhR was recruited to the *Cyp19* promoter in vivo. As the *Cyp19* gene is synergistically activated by AhR/Arnt and Ad4BP/SF-1, we assumed that AhR, Arnt, and Ad4BP/SF-1 physically interact on the *Cyp19* promoter. We next examined whether anti-AhR antibodies precipitate

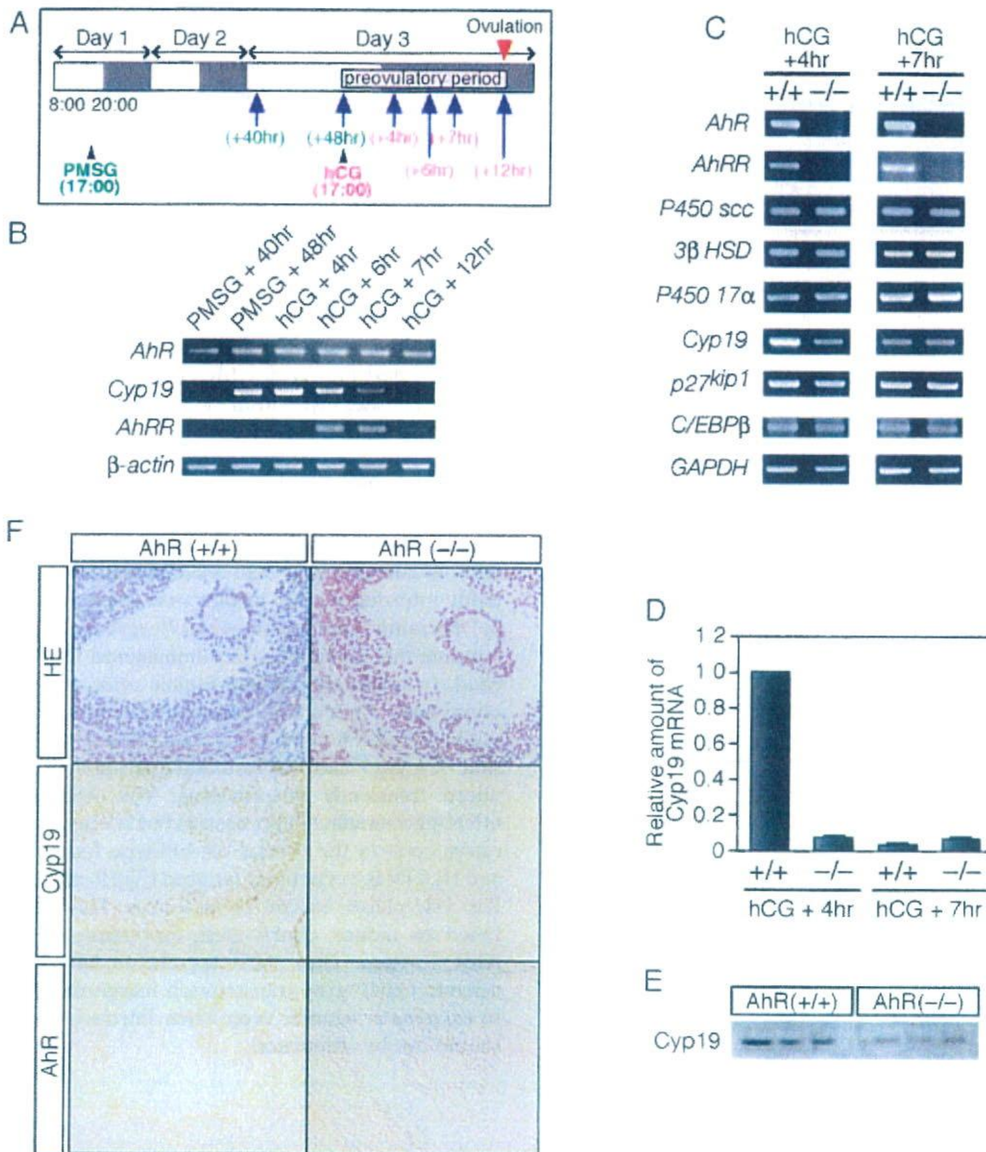
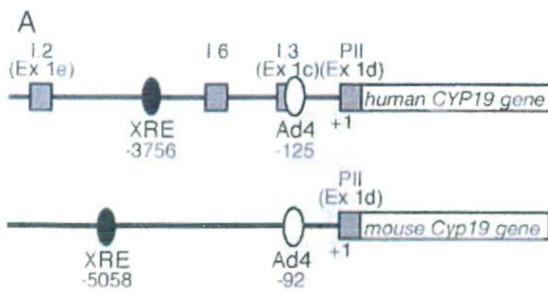
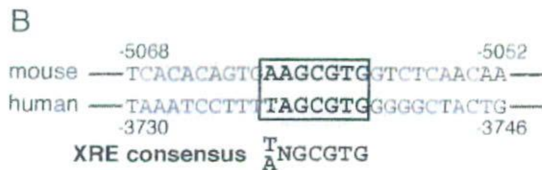
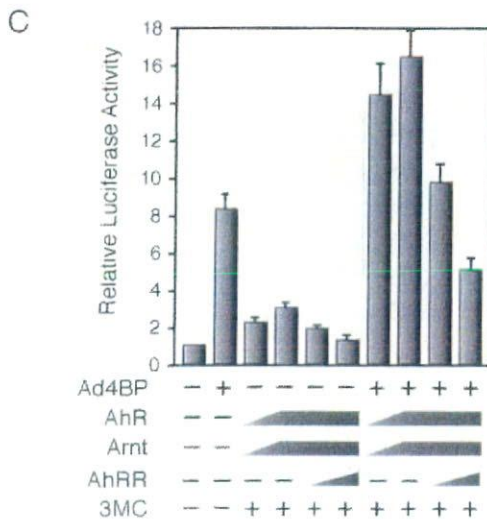


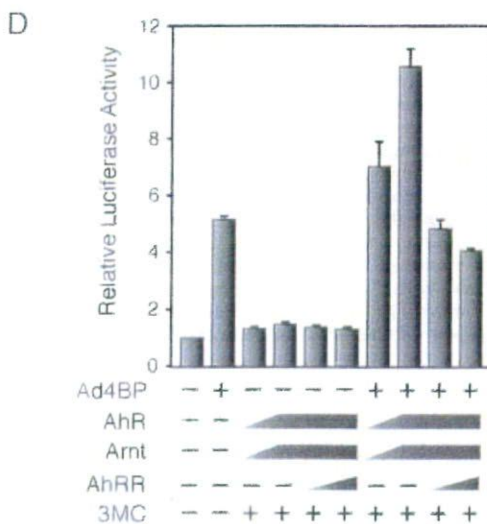
FIG. 4. AhR regulates the expression of ovarian *Cyp19* during the preovulatory period. (A) Schematic representation of the experimental procedure. The estrus cycle was induced artificially by intraperitoneal injection of PMSG at 1700 h on day 1 and of hCG at 1700 h on day 3. Ovaries were collected 40 and 48 h after PMSG injection or 4, 6, 7, and 12 h after hCG injection (indicated by arrows). (B) Profiles of mRNA expression for AhR, AhRR, and Cyp19 during the preovulatory period. Total RNA samples, prepared from ovaries derived from hormone-treated mice at the indicated times (top), were subjected to RT-PCR with primers sets specific for AhR, AhRR, and Cyp19. β-Actin mRNA was used as a control. (C) Expression of mRNAs encoding steroidogenic enzymes and proteins involved in ovarian folliculogenesis. Total RNA samples, prepared from the ovaries of hormone-treated AhR$^{+/+}$ and AhR$^{-/-}$ mice at the indicated times (top), were used for RT-PCR with the PCR primers. (D) Quantification of Cyp19 mRNA levels. Total RNA samples, prepared from the ovaries isolated 4 and 7 h after hCG injection, were subjected to quantitative RT-PCR analyses. Three animals were used for this experiment. (E) Expression of Cyp19 protein within AhR$^{+/+}$ and AhR$^{-/-}$ ovaries during the preovulatory period. Whole-cell extracts (10 μg), prepared from the ovaries of hormone-treated (hCG + 5 h) mice, were subjected to Western blot analysis with an anti-Cyp19 antibody. Three AhR$^{+/+}$ and three AhR$^{-/-}$ animals were used for these experiments. (F) Immunohistochemical staining of Cyp19 and AhR in the granulosa cells of AhR$^{+/+}$ and AhR$^{-/-}$ ovaries. Five-micrometer paraffin sections were prepared from the ovaries of hormone-treated (hCG + 5 h) mice. Sections were stained with hematoxylin-eosin (HE) or with anti-AhR or anti-Cyp19 antibody.

the Ad4 site of the *Cyp19* promoter and whether the anti-Ad4BP/SF-1 antibody reciprocally precipitates the XRE sequence. Both the XRE- and Ad4-containing sequences of the *Cyp19* promoter were recovered in both anti-Ad4BP/SF-1 and anti-AhR immunoprecipitates (Fig. 6D). As a control, the sequence between bp −2740 and −2441 was not recovered in either the anti-AhR or the anti-Ad4BP/SF-1 immunoprecipitate, excluding the possibility that incomplete fragmentation of









DNA during chromatin preparation resulted in artifactual coimmunoprecipitation of the Ad4- and XRE-containing sequences. To confirm the interaction between AhR and Ad4BP/SF-1 on the *Cyp19* promoter, we investigated whether the XRE is coimmunoprecipitated with Ad4BP/SF-1 in the $AhR^{-/-}$ chromatin (Fig. 6E). Anti-Ad4BP antibody failed to precipitate the XRE-containing sequence in the absence of AhR, indicating that Ad4BP/SF-1 does not bind directly to the XRE but binds indirectly through interaction with the XRE-bound AhR. In addition, we investigated whether AhR knockout affects Ad4BP/SF-1 binding to the Ad4 site and found that there is no difference in binding of Ad4BP/SF-1 between $AhR^{+/+}$ and $AhR^{-/-}$ mice (Fig. 6E). These results clearly demonstrated that both AhR and Ad4BP/SF-1 bind to their cognate binding sites within the *Cyp19* promoter and physically interact, probably leading to cooperative enhancement of *Cyp19* expression.

AhR ligands exerted an estrogenic effect by aberrantly activating *Cyp19* gene expression. While *Cyp19* is expressed transiently at a particular time point in the preovulatory period, AhR is constitutively expressed in granulosa cells. Inadvertently introduced AhR ligands may exert an estrogenic effect by aberrantly upregulating *Cyp19* expression in the ovary. To examine this possibility, we administered DMBA, an AhR ligand, to randomly selected female mice regardless of estrus cycle phase. After a 5-h treatment, we examined the expression of Cyp19 mRNA in the ovary. In a normal estrus cycle, expression of Cyp19 and the resultant estradiol production are induced transiently at proestrus. We observed that Cyp19 mRNA accumulated at proestrus but not at other phases of the estrus cycle in the ovaries of wild-type female mice (Fig. 7A and B). DMBA effectively induced *Cyp19* expression at most of the cycle phases except for metestrus. This reagent, however, failed to induce *Cyp19* gene expression in the ovaries of $AhR^{-/-}$ mice. Thus, AhR appears to have the potential to activate *Cyp19* gene transcription inappropriately in response to exogenous ligands, even when intrinsic estrogen synthesis should not be stimulated.

FIG. 5. Cooperative activation of AhR and Ad4BP/SF-1 on the *Cyp19/CYP19* promoter. (A) Schematic representation of the mouse *Cyp19* and human *CYP19* gene promoter regions. The square boxes indicate the first exons, exons I.2 (Ex 1e), I.6, I.3 (Ex 1c), and PII (Ex 1d), expressed specifically in the placenta, bone, adipose tissue, and ovary, respectively. The filled and open ovals represent the AhR/Arnt-binding (XRE) and Ad4BP/SF-1-binding (Ad4) sequences, respectively. The ovary-specific transcription start site is numbered as +1, and the positions of the XRE and Ad4 sites were numbered as the negative values of their distances from the transcription start site. (B) Nucleotide sequences containing the XRE site from the mouse *Cyp19* and human *CYP19* gene upstream regions. The consensus XRE sequence is indicated in bold letters. (C) Cooperative activation of AhR and Ad4BP/SF-1 on the human *CYP19* gene promoter. Expression plasmids encoding AhR, Arnt, AhRR, and Ad4BP/SF-1 were cotransfected into 293 cells with a reporter plasmid, in which luciferase expression is driven by the *CYP19* promoter (h*CYP19*-3853Luc), in the presence (+) or absence (−) of 3MC. After a 48-h incubation, cells were recovered and subjected to luciferase assays. All values are the means ± SD for three experiments. (D) Cooperative activation of AhR and Ad4BP/SF-1 on the mouse *Cyp19* promoter. m*Cyp19*-5335Luc was used for this assay. All other conditions were as specified for panel C.

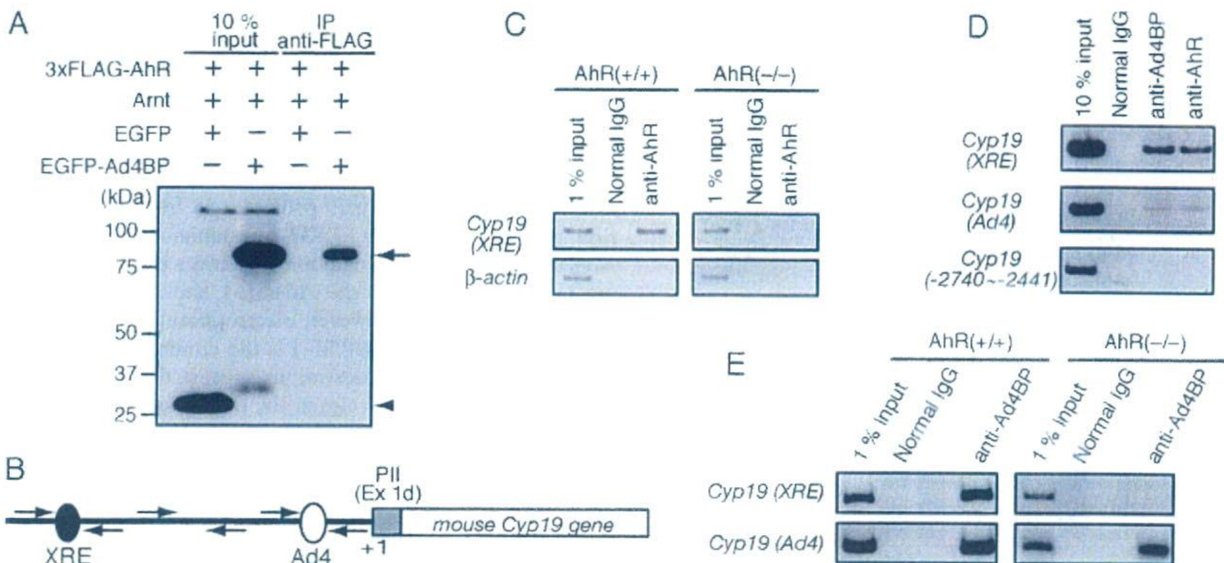


FIG. 6. Interaction of AhR with Ad4BP/SF-1 on the *Cyp19* promoter. (A) Detection of a physical interaction between AhR and Ad4BP/SF-1 by coimmunoprecipitation. FLAG-tagged proteins from whole-cell extracts of 293 cells transfected with 3×FLAG-AhR, Arnt, and EGFP-Ad4BP were immunoprecipitated (IP) with an anti-FLAG antibody. The immunoprecipitates were then subjected to immunoblotting with an anti-GFP antibody. An EGFP expression vector was transfected as a control. An arrow and an arrowhead indicate the EGFP-Ad4BP and EGFP samples, respectively. (B) Schematic representation of the location of primers used in the ChIP assays. Three sets of primers were used to amplify DNA regions containing the XRE site at −5058 and the Ad4/SF-1 sequence at −92 and a third unrelated region (−2740 to −2441), containing neither of them, as a control. (C) Binding of AhR to the promoter region of the *Cyp19* gene, revealed by ChIP assays. Soluble chromatin, prepared from preovulatory granulosa cells (hCG + 2 h), was subjected to ChIP assay with an anti-AhR antibody. β-Actin was used as a negative control. (D) Interaction between AhR and Ad4BP/SF-1 on the *Cyp19* gene promoter. Chromatin isolated from preovulatory granulosa cells was incubated with anti-AhR or anti-Ad4BP/SF-1 antibody and then subjected to PCR with two sets of primers amplifying the XRE and Ad4 sites. A primer pair specific for the sequence from −2740 to about −2441 was used as a control. (E) Binding of Ad4BP/SF-1 to the XRE and Ad4 sites in the presence or absence of AhR, revealed by ChIP assays. Chromatin isolated from preovulatory granulosa cells of the $AhR^{+/+}$ and $AhR^{-/-}$ ovaries was incubated with anti-Ad4BP/SF-1 or control antibody and then subjected to PCR to amplify the XRE and Ad4 sites. IgG, immunoglobulin G.

DISCUSSION

In agreement with a previous report (1), $AhR^{-/-}$ females demonstrated compromised fertility. The number of delivered pups was clearly decreased in comparison to those delivered by wild-type animals. As the phenotype of *AhR* gene disruption suggested a novel physiological function for AhR, in addition to its well-established xenobiotic metabolizing function, we investigated the molecular mechanisms underlying defective fertility in $AhR^{-/-}$ female mice.

Reproductive defects seen with $AhR^{-/-}$ female are primarily due to insufficient synthesis of estradiol in the ovary. Abbott et al. described that $AhR^{-/-}$ females exhibited difficulties in maintaining conceptuses during pregnancy (1), while Benedict et al. reported that AhR deficiency affected follicular maturation and ovulation under normal growth conditions (3, 4). Our studies indicated that follicles present in the ovaries of $AhR^{-/-}$ mice developed to an antral/preovulatory stage, while the corpus luteum was barely detectable. Upon stimulation of superovulation, the number of ovulated oocytes in $AhR^{-/-}$ females was significantly lower than those seen with the wild type. In conjunction with the observations of Benedict et al., these results suggested that the reduced fertility of $AhR^{-/-}$ females was a consequence of ovarian defects during the period of late folliculogenesis to follicular rupture.

Both implantation and follicular maturation are highly dependent on estrogenic action (12). The phenotype of $AhR^{-/-}$ mice suggested the hypothesis that the observed reproductive failure might be induced by the disruption of genes involved in estrogen production or action. The ovaries of ArKO mice were reported to contain many large follicles filled with granulosa cells, with an absence of a corpus luteum (17). ERαβKO female mice (14), completely lacking a receptor-mediated response to estrogen, failed to induce preovulatory follicle formation after superovulation treatment. The female reproduction defects of ArKO and ERαβKO mice resembled those of AhR KO mice, albeit with a more severe phenotype. The similarities between these phenotypes strongly suggested that AhR KO mice have deficits in estrogen production or action. After hypothesizing that estradiol production in the preovulatory period was affected in AhR KO females, we determined that intraovarian estrogen concentrations during the preovulatory stages were decreased in $AhR^{-/-}$ females. Administration of estradiol increased the number of ovulated oocytes in $AhR^{-/-}$ females, suggesting that the subfertility of $AhR^{-/-}$ mice results primarily from reduced levels of ovarian estrogen.

***Cyp19* gene transcription mediated synergistically by AhR and Ad4BP/SF-1.** Ovarian sex steroids, such as estrogen and progesterone, are synthesized from cholesterol through multi-

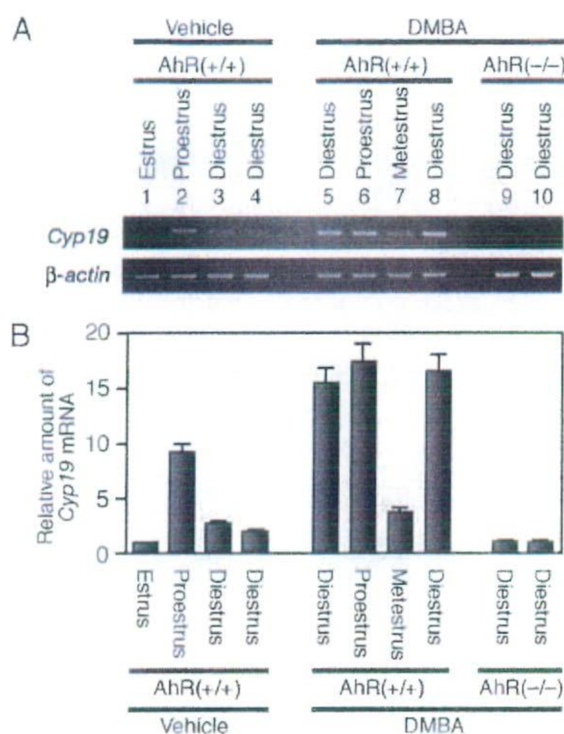


FIG. 7. Upregulation of *Cyp19* expression by an exogenous AhR ligand, DMBA. (A) Expression of *Cyp19* induced by intraperitoneal injection of DMBA. $AhR^{+/+}$ (lanes 1 to 8) or $AhR^{-/-}$ (lanes 9 and 10) female mice were injected intraperitoneally with DMBA (50 mg/kg of body weight) or vehicle alone. Five hours after injection, we prepared total RNA from the ovaries. The amounts of *Cyp19* mRNA were then evaluated by RT-PCR. The estrus cycle phase of each animal was determined by observing vaginal smears collected just before injection of DMBA. β-Actin was used as a control. (B) Quantitative representation of *Cyp19* mRNA levels. Quantification of the *Cyp19* transcript was performed by using a 7500 real-time PCR system (Applied Biosystems, Japan).

ple reactions in the ovary. Investigation of steroidogenic gene expression revealed that *Cyp19* expression was significantly reduced in $AhR^{-/-}$ females. Immunohistochemical and immunoblotting analyses confirmed the reduced levels of Cyp19 in granulosa cells. As Cyp19 is the rate-limiting enzyme in estrogen synthesis, it is likely that the reduced estradiol concentrations result primarily from lower levels of Cyp19 synthesis in the ovaries of $AhR^{-/-}$ females.

The *Cyp19* gene has multiple tissue-specific first exons (23, 33, 55). A survey of the 5′ sequence upstream of the ovary-specific first exon revealed the presence of a potential XRE sequence in both human and mouse genes. The presence of such an XRE sequence has recently been reported to occur within the promoter of the fish ovarian-type *CYP19* genes, although a functional analysis remains to be performed (7, 27, 59, 60). The conservation of XRE among a variety of animal species, however, suggests functionality of this sequence in the ovary-specific expression of *Cyp19*. In this study, we substantiated this hypothesis by transient transfection and ChIP assays. In addition, the *Cyp19* gene proximal promoter contained a functional Ad4/SF-1 site (32). Our investigation of the functional correlation between Ad4BP/SF-1 and AhR revealed that these factors cooperatively enhanced *Cyp19* gene transcription. This synergistic action resulted from a physical interaction, revealed by coimmunoprecipitation and ChIP assays.

Recently, another orphan nuclear receptor, LRH-1 (*l*iver *r*eceptor *h*omologue 1), has been reported to be selectively expressed in ovarian granulosa cells (15, 24) and to transactivate the ovary-specific *Cyp19* promoter in transient transfection assays. Structurally, LRH-1 exhibits homology with Ad4BP/SF-1, and the recognition sequences of these proteins are quite similar. Using the Ad4/SF-1 site from the *Cyp19* promoter as a probe, however, electrophoretic mobility shift assays revealed that Ad4BP/SF-1 is the dominant binding factor (9, 10, 15). These observations suggest that Ad4BP/SF-1 and LRH-1 play distinct roles in the regulation of target gene transcription. As LRH-1 is involved in cell proliferation via regulation of cyclin D1 and E1 gene expression (5), further investigations are needed to clarify the function of LRH-1 in the AhR-mediated expression of *Cyp19* in the ovary.

Role of negative feedback regulatory loop formed by AhR and AhRR. AhRR is one of the downstream targets of AhR transcriptional regulation (2, 36). Structurally, AhRR belongs to a family of bHLH-PAS transcription factors and suppresses AhR-mediated transactivation by competing with AhR for heterodimer formation with Arnt. This study confirmed the suppressive function of AhRR on *Cyp19* gene expression. The expressions of both AhRR and *Cyp19* are similarly regulated by AhR via binding of AhR to the XRE sequences in their promoters. Superovulation experiments, however, revealed that the *Cyp19* gene displayed an earlier peak of expression (reaching a maximum at 48 to 52 h after gonadotropin [PMSG] treatment) than AhRR, which was upregulated as *Cyp19* expression began to decline. Although the mechanisms producing this time lag of AhRR expression are unknown, cyclic expression of *Cyp19* in the estrus cycle could be explained by a lag in AhRR synthesis. From these observations, it is possible that AhR and AhRR regulate the ovarian biological clock governing the estrus cycle. In support of this possibility, it is interesting to note that expression of CLOCK and BMAL1 (Arnt3), two members of the bHLH-PAS family (to which AhR and AhRR belong), in the suprachiasmatic nuclei of the hypothalamus regulates the expression of their inhibitors, PERs, to generate the biological clock governing circadian rhythms (31).

In a normal ovarian cycle, the expression levels of AhR appear to be constant. Thus, to transactivate the expression of *Cyp19* and AhRR, AhR may also need to be activated. Although a number of endogenous ligands have been reported to activate AhR (13), the identity of the endogenous ligand required for the activation of AhR in the ovary and the mechanism by which this activation occurs during the estrus cycle remains unknown. In keratinocyte cell lines cultured at low density or in Ca^{2+}-free medium, AhR translocates to the nucleus to activate reporter genes, even in the absence of obvious AhR ligands (25). The activation of AhR by phosphorylation has been suggested for such cases (26).

Estrogenic effect of AhR ligand through two distinct mechanisms. In this study, we characterized the subfertility phenotype of $AhR^{-/-}$ female mice, identifying the key role of AhR in *Cyp19* gene transcription controlling the temporal synthesis

of ovarian estrogen in the estrus cycle. This intrinsic physiological role of AhR provides an explanation for the high degree of AhR conservation throughout vertebrate species. This finding also provides a molecular basis for the estrogenic actions of AhR ligands. DMBA, an AhR ligand, induced *Cyp19* expression, leading to unscheduled increases in estradiol regardless of estrus cycle phase. Recently, functional cross talk was reported between AhR and estrogen receptors (ER) (44), and the ligand-bound AhR exerts estrogenic effects through a direct interaction with nonliganded ER molecules associated with estrogen response elements in target gene promoters. Together with our observations, ligand-bound AhR likely exerts an estrogenic effect via two distinct mechanisms, the stimulation of estradiol production through the activation of *Cyp19* gene expression and the activation of empty ER by AhR coactivation.

ACKNOWLEDGMENTS

We thank R. S. Pollenz (University of South Florida) for kindly providing the AhR antibody and T. Etoh (Central Laboratories for Experimental Animals, Japan) for the care of laboratory mice. We are also grateful to Y. Nemoto for clerical work.

This work was funded in part by Core Research for Evolutionary Science and Technology, Solution Oriented Research for Science and Technology from Japan Science and Technology and Research Fellowship (T. Baba) of the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. **Abbott, B. D., J. E. Schmid, J. A. Pitt, A. R. Buckalew, C. R. Wood, G. A. Held, and J. J. Diliberto.** 1999. Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. Toxicol. Appl. Pharmacol. **155:**62–70.
2. **Baba, T., J. Mimura, K. Gradin, A. Kuroiwa, T. Watanabe, Y. Matsuda, J. Inazawa, K. Sogawa, and Y. Fujii-Kuriyama.** 2001. Structure and expression of the Ah receptor repressor gene. J. Biol. Chem. **276:**33101–33110.
3. **Benedict, J. C., T. M. Lin, I. K. Loeffler, R. E. Peterson, and J. A. Flaws.** 2000. Physiological role of the aryl hydrocarbon receptor in mouse ovary development. Toxicol. Sci. **56:**382–388.
4. **Benedict, J. C., K. P. Miller, T. M. Lin, C. Greenfeld, J. K. Babus, R. E. Peterson, and J. A. Flaws.** 2003. Aryl hydrocarbon receptor regulates growth, but not atresia, of mouse preantral and antral follicles. Biol. Reprod. **68:** 1511–1517.
5. **Botrugno, O. A., E. Fayard, J. S. Annicotte, C. Haby, T. Brennan, O. Wendling, T. Tanaka, T. Kodama, W. Thomas, J. Auwerx, and K. Schoonjans.** 2004. Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. Mol. Cell **15:**499–509.
6. **Brown, N. M., P. A. Manzolillo, J. X. Zhang, J. Wang, and C. A. Lamartiniere.** 1998. Prenatal TCDD and predisposition to mammary cancer in the rat. Carcinogenesis **19:**1623–1629.
7. **Callard, G. V., A. V. Tchoudakova, M. Kishida, and E. Wood.** 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. J. Steroid Biochem. Mol. Biol. **79:**305–314.
8. **Carlone, D. L., and J. S. Richards.** 1997. Functional interactions, phosphorylation, and levels of 3′,5′-cyclic adenosine monophosphate-regulatory element binding protein and steroidogenic factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. Mol. Endocrinol. **11:**292–304.
9. **Clyne, C. D., A. Kovacic, C. J. Speed, J. Zhou, V. Pezzi, and E. R. Simpson.** 2004. Regulation of aromatase expression by the nuclear receptor LRH-1 in adipose tissue. Mol. Cell. Endocrinol. **215:**39–44.
10. **Clyne, C. D., C. J. Speed, J. Zhou, and E. R. Simpson.** 2002. Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes. J. Biol. Chem. **277:**20591–20597.
11. **Conn, P. M., J. G. Chafouleas, D. Rogers, and A. R. Means.** 1981. Gonadotropin releasing hormone stimulates calmodulin redistribution in rat pituitary. Nature **292:**264–265.
12. **Curtis Hewitt, S., E. H. Goulding, E. M. Eddy, and K. S. Korach.** 2002. Studies using the estrogen receptor alpha knockout uterus demonstrate that implantation but not decidualization-associated signaling is estrogen dependent. Biol. Reprod. **67:**1268–1277.
13. **Denison, M. S., and S. R. Nagy.** 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. **43:**309–334.
14. **Dupont, S., A. Krust, A. Gansmuller, A. Dierich, P. Chambon, and M. Mark.** 2000. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development **127:**4277–4291.
15. **Falender, A. E., R. Lanz, D. Malenfant, L. Belanger, and J. S. Richards.** 2003. Differential expression of steroidogenic factor-1 and FTF/LRH-1 in the rodent ovary. Endocrinology **144:**3598–3610.
16. **Fero, M. L., M. Rivkin, M. Tasch, P. Porter, C. E. Carow, E. Firpo, K. Polyak, L. H. Tsai, V. Broudy, R. M. Perlmutter, K. Kaushansky, and J. M. Roberts.** 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell **85:**733–744.
17. **Fisher, C. R., K. H. Graves, A. F. Parlow, and E. R. Simpson.** 1998. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. Proc. Natl. Acad. Sci. USA **95:**6965–6970.
18. **Fujisawa-Sehara, A., K. Sogawa, M. Yamane, and Y. Fujii-Kuriyama.** 1987. Characterization of xenobiotic responsive elements upstream from the drug-metabolizing cytochrome P-450c gene: a similarity to glucocorticoid regulatory elements. Nucleic Acids Res. **15:**4179–4191.
19. **Gibbons, A.** 1993. Dioxin tied to endometriosis. Science **262:**1373.
20. **Hahn, M. E.** 2002. Aryl hydrocarbon receptors: diversity and evolution. Chem.-Biol. Interact. **141:**131–160.
21. **Hankinson, O.** 1995. The aryl hydrocarbon receptor complex. Annu. Rev. Pharmacol. Toxicol. **35:**307–340.
22. **Harada, N.** 1988. Novel properties of human placental aromatase as cytochrome P-450: purification and characterization of a unique form of aromatase. J. Biochem. (Tokyo) **103:**106–113.
23. **Harada, N., T. Utsumi, and Y. Takagi.** 1993. Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proc. Natl. Acad. Sci. USA **90:**11312–11316.
24. **Hinshelwood, M. M., J. J. Repa, J. M. Shelton, J. A. Richardson, D. J. Mangelsdorf, and C. R. Mendelson.** 2003. Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cell types correlates with differing function. Mol. Cell. Endocrinol. **207:**39–45.
25. **Ikuta, T., Y. Kobayashi, and K. Kawajiri.** 2004. Cell density regulates intracellular localization of aryl hydrocarbon receptor. J. Biol. Chem. **279:** 19209–19216.
26. **Ikuta, T., Y. Kobayashi, and K. Kawajiri.** 2004. Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. Biochem. Biophys. Res. Commun. **317:**545–550.
27. **Kazeto, Y., S. Ijiri, A. R. Place, Y. Zohar, and J. M. Trant.** 2001. The 5′-flanking regions of CYP19A1 and CYP19A2 in zebrafish. Biochem. Biophys. Res. Commun. **288:**503–508.
28. **Kazlauskas, A., L. Poellinger, and I. Pongratz.** 1999. Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (aryl hydrocarbon) receptor. J. Biol. Chem. **274:**13519–13524.
29. **Kiyokawa, H., R. D. Kineman, K. O. Manova-Todorova, V. C. Soares, E. S. Hoffman, M. Ono, D. Khanam, A. C. Hayday, L. A. Frohman, and A. Koff.** 1996. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell **85:**721–732.
30. **Komatsu, T., H. Mizusaki, T. Mukai, H. Ogawa, D. Baba, M. Shirakawa, S. Hatakeyama, K. I. Nakayama, H. Yamamoto, A. Kikuchi, and K. Morohashi.** 2004. Small ubiquitin-like modifier 1 (SUMO-1) modification of the synergy control motif of Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) regulates synergistic transcription between Ad4BP/SF-1 and Sox9. Mol. Endocrinol. **18:**2451–2462.
31. **Lowrey, P. L., and J. S. Takahashi.** 2000. Genetics of the mammalian circadian system: photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. Annu. Rev. Genet. **34:**533–562.
32. **Lynch, J. P., D. S. Lala, J. J. Peluso, W. Luo, K. L. Parker, and B. A. White.** 1993. Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. Mol. Endocrinol. **7:**776–786.
33. **Means, G. D., M. W. Kilgore, M. S. Mahendroo, C. R. Mendelson, and E. R. Simpson.** 1991. Tissue-specific promoters regulate aromatase cytochrome P450 gene expression in human ovary and fetal tissues. Mol. Endocrinol. **5:**2005–2013.
34. **Meyer, B. K., and G. H. Perdew.** 1999. Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization. Biochemistry **38:**8907–8917.
35. **Meyer, B. K., M. G. Pray-Grant, J. P. Vanden Heuvel, and G. H. Perdew.** 1998. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol. Cell. Biol. **18:**978–988.
36. **Mimura, J., M. Ema, K. Sogawa, and Y. Fujii-Kuriyama.** 1999. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. Genes Dev. **13:**20–25.
37. **Mimura, J., and Y. Fujii-Kuriyama.** 2003. Functional role of AhR in the expression of toxic effects by TCDD. Biochim. Biophys. Acta **1619:**263–268.
38. **Mimura, J., K. Yamashita, K. Nakamura, M. Morita, T. N. Takagi, K. Nakao, M. Ema, K. Sogawa, M. Yasuda, M. Katsuki, and Y. Fujii-Kuriyama.**

1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells **2**:645–654.

39. **Mizusaki, H., K. Kawabe, T. Mukai, E. Ariyoshi, M. Kasahara, H. Yoshioka, A. Swain, and K. Morohashi.** 2003. *Dax-1* (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) gene transcription is regulated by Wnt4 in the female developing gonad. Mol. Endocrinol. **17**:507–519.
40. **Morohashi, K., S. Honda, Y. Inomata, H. Handa, and T. Omura.** 1992. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J. Biol. Chem. **267**:17913–17919.
41. **Morohashi, K., U. M. Zanger, S. Honda, M. Hara, M. R. Waterman, and T. Omura.** 1993. Activation of CYP11A and CYP11B gene promoters by the steroidogenic cell-specific transcription factor, Ad4BP. Mol. Endocrinol. **7**:1196–1204.
42. **Nelson, J. F., L. S. Felicio, P. K. Randall, C. Sims, and C. E. Finch.** 1982. A longitudinal study of estrous cyclicity in aging C57BL/6J mice. I. Cycle frequency, length and vaginal cytology. Biol. Reprod. **27**:327–339.
43. **Nomura, M., K. Kawabe, S. Matsushita, S. Oka, O. Hatano, N. Harada, H. Nawata, and K. Morohashi.** 1998. Adrenocortical and gonadal expression of the mammalian Ftz-F1 gene encoding Ad4BP/SF-1 is independent of pituitary control. J. Biochem. (Tokyo) **124**:217–224.
44. **Ohtake, F., K. Takeyama, T. Matsumoto, H. Kitagawa, Y. Yamamoto, K. Nohara, C. Tohyama, A. Krust, J. Mimura, P. Chambon, J. Yanagisawa, Y. Fujii-Kuriyama, and S. Kato.** 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature **423**: 545–550.
45. **Omura, T., and K. Morohashi.** 1995. Gene regulation of steroidogenesis. J. Steroid Biochem. Mol. Biol. **53**:19–25.
46. **Orlando, V., H. Strutt, and R. Paro.** 1997. Analysis of chromatin structure by in vivo formaldehyde cross-linking. Methods **11**:205–214.
47. **Pall, M., P. Hellberg, M. Brannstrom, M. Mikuni, C. M. Peterson, K. Sundfeldt, B. Norden, L. Hedin, and S. Enerback.** 1997. The transcription factor C/EBP-beta and its role in ovarian function; evidence for direct involvement in the ovulatory process. EMBO J. **16**:5273–5279.
48. **Parekh, B. S., and T. Maniatis.** 1999. Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. Mol. Cell **3**:125–129.
49. **Poland, A., E. Glover, and A. S. Kende.** 1976. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. J. Biol. Chem. **251**:4936–4946.
50. **Poland, A., and J. C. Knutson.** 1982. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu. Rev. Pharmacol. Toxicol. **22**:517–554.
51. **Richards, J. S.** 1994. Hormonal control of gene expression in the ovary. Endocr. Rev. **15**:725–751.
52. **Richards, J. S.** 2001. Perspective: the ovarian follicle—a perspective in 2001. Endocrinology **142**:2184–2193.
53. **Robles, R., Y. Morita, K. K. Mann, G. I. Perez, S. Yang, T. Matikainen, D. H. Sherr, and J. L. Tilly.** 2000. The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. Endocrinology **141**:450–453.
54. **Roby, K. F.** 2001. Alterations in follicle development, steroidogenesis, and gonadotropin receptor binding in a model of ovulatory blockade. Endocrinology **142**:2328–2335.
55. **Sebastian, S., and S. E. Bulun.** 2001. A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project. J. Clin. Endocrinol. Metab. **86**:4600–4602.
56. **Shimizu, Y., Y. Nakatsuru, M. Ichinose, Y. Takahashi, H. Kume, J. Mimura, Y. Fujii-Kuriyama, and T. Ishikawa.** 2000. Benzo[*a*]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proc. Natl. Acad. Sci. USA **97**:779–782.
57. **Stanislaus, D., J. A. Janovick, T. Ji, T. M. Wilkie, S. Offermanns, and P. M. Conn.** 1998. Gonadotropin and gonadal steroid release in response to a gonadotropin-releasing hormone agonist in Gqalpha and G11alpha knockout mice. Endocrinology **139**:2710–2717.
58. **Sterneck, E., L. Tessarollo, and P. F. Johnson.** 1997. An essential role for C/EBPbeta in female reproduction. Genes Dev. **11**:2153–2162.
59. **Tchoudakova, A., M. Kishida, E. Wood, and G. V. Callard.** 2001. Promoter characteristics of two *cyp19* genes differentially expressed in the brain and ovary of teleost fish. J. Steroid Biochem. Mol. Biol. **78**:427–439.
60. **Tong, S. K., and B. C. Chung.** 2003. Analysis of zebrafish *cyp19* promoters. J. Steroid Biochem. Mol. Biol. **86**:381–386.
61. **Whitelaw, M., I. Pongratz, A. Wilhelmsson, J.-Å. Gustafsson, and L. Poellinger.** 1993. Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor. Mol. Cell. Biol. **13**: 2504–2514.
62. **Yoshinaga-Hirabayashi, T., K. Ishimura, H. Fujita, J. Kitawaki, and Y. Osawa.** 1990. Immunocytochemical localization of aromatase in immature rat ovaries treated with PMSG and hCG, and in pregnant rat ovaries. Histochemistry **93**:223–228.

Molecular and Cellular Biology, Nov. 2005, p. 9360–9368 Vol. 25, No. 21
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.21.9360–9368.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Constitutive Expression of Aryl Hydrocarbon Receptor in Keratinocytes Causes Inflammatory Skin Lesions

Masafumi Tauchi,[1,2] Azumi Hida,[1,2] Takaaki Negishi,[3] Fumiki Katsuoka,[1,2] Shuhei Noda,[1,2] Junsei Mimura,[1,2,6] Tomonori Hosoya,[4] Akinori Yanaka,[1] Hiroyuki Aburatani,[5] Yoshiaki Fujii-Kuriyama,[2,6] Hozumi Motohashi,[1,2]* and Masayuki Yamamoto[1,2,4]

Graduate School of Comprehensive Human Sciences[1] and Center for Tsukuba Advanced Research Alliance,[2] University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Mochida Pharmaceutical Co., Ltd., Pharmaceutical Research Center, 722 Uenohara, Jimba, Gotemba 412-8524,[3] ERATO Environmental Response Project, Japan Science and Technology Corporation, 1-1-1 Tennoudai, Tsukuba 305-8577,[4] Research Center for Advance Science and Technology, The University of Tokyo, Tokyo 153-8904,[5] and SORST, Japan Science and Technology Corporation, Kawaguchi 332-0012,[6] Japan

Received 23 May 2005/Returned for modification 25 June 2005/Accepted 7 August 2005

Occupational and environmental exposure to polycyclic aromatic hydrocarbons (PAHs) has been suggested to provoke inflammatory and/or allergic disorders, including asthma, rhinitis, and dermatitis. The molecular mechanisms of this PAH-mediated inflammation remain to be clarified. Previous studies implied the involvement of PAHs as irritants and allergens, with the reactive oxygen species generated from the oxygenated PAHs believed to be an exacerbating factor. It is also possible that PAHs contribute to the pathogenesis through activation of aryl-hydrocarbon receptor (AhR)-mediated transcription, since PAHs are potent inducers of the AhR. To address this point, we generated transgenic mouse lines expressing the constitutive active form of the AhR in keratinocytes. In these lines of mice, the AhR activity was constitutively enhanced in the absence of ligands, so that any other direct effects of PAHs and their metabolites could be ignored. At birth, these transgenic mice were normal, but severe skin lesions with itching developed postnatally. The skin lesions were accompanied by inflammation and immunological imbalance and resembled typical atopic dermatitis. We demonstrate that constitutive activation of the AhR pathway causes inflammatory skin lesions and suggests a new mechanism for the exacerbation of inflammatory diseases after exposure to occupational and environmental xenobiotics.

A steady increase in the prevalence of allergic diseases has been noted over the last century (30). Exposure to environmental xenobiotics was reported as one of the risk factors associated with the development of atopy and asthma (3). Polycyclic aromatic hydrocarbons (PAHs) are one of major environmental pollutants present in automobile exhaust, cigarette smoke, various foods, and industrial wastes. The skin, respiratory tract, and digestive tract are the first tissues that come into contact with these exogenous chemicals. Recent studies suggested that inhalation of PAHs in diesel exhaust particles and cigarette smoke triggers inflammatory responses in the respiratory tract, resulting in rhinitis and asthma (13, 21, 31, 33). Occupational exposure to PAHs or topical application of chemicals or drugs containing PAHs elicits inflammatory skin diseases, known as contact hypersensitivity or dermatitis (36, 37). In spite of the increasing number of reports showing a relationship between PAHs and inflammatory disorders, the precise molecular mechanisms by which such chemicals contribute to the development of pathological states remain to be clarified.

The carcinogenic and mutagenic effects of PAHs are well documented (for instance, see review in reference 32) and genetic and biochemical studies indicate that most of these responses elicited by PAHs are mediated through binding to aryl-hydrocarbon receptor (AhR) (22), since PAHs are potent inducers of the AhR activity (12). On the contrary, the involvement of AhR in the inflammatory effect of PAHs is controversial, since dioxins, a typical group of ligands for AhR, suppress the allergen-specific immune responses (10, 35) and often induce chloracne, whose clinical and histopathological appearance is rather distinct from those of PAH-mediated contact dermatitis (6, 37). Usually, the latter is accompanied by itching and is associated with inflammation, whereas the former does not display these signs even in the late stages of the disease (6, 37). Instead, the PAHs have been suggested to provoke inflammation as primary irritants or by allergic mechanisms against PAHs or their metabolites (1, 7, 37). Other lines of evidence suggest that reactive oxygen species generated by oxygenated PAHs appear to enhance the allergic response (4, 15). PAHs have also been shown to stimulate an increase in the DNA-binding activity of NF-κB, which in turn induces cytokine gene expression and provokes the allergic response (28).

The effect of environmental xenobiotics on the immune systems has been intensively examined, but uncertainty remains as to whether these compounds do indeed influence immune responses. Difference in the experimental systems seems to

* Corresponding author. Mailing address: Center for TARA, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan. Phone: 81-29-853-7320. Fax: 81-29-853-7318. E-mail: hozumim@tara.tsukuba.ac.jp.

give rise to distinct results. For instance, there is a report showing that PAHs and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) directly enhance the immunoglobulin E (IgE) production when added to the purified B cells (33). Another study showed that TCDD preincubation results in the decrease of IgE production from the B-cell culture (14). This situation convinced us that an integrated in vivo experimental system should be established for evaluating and dissecting the biological effect of these compounds.

We surmised that, in any case, AhR-mediated transcription should be activated in the tissues where the AhR ligands are applied. In order to clarify the contribution of AhR-mediated transcription to the development of inflammatory disorders, we attempted to discriminate between the contribution of the AhR pathway and the other effects of PAHs. Therefore, in the present study, we assessed the direct contribution of the AhR pathway to inflammatory disorders by utilizing the constitutive active form of AhR (AhR-CA) that can activate transcription in the absence of exogenous chemicals. Skin was chosen as the tissue to examine AhR-CA expression because the consequences are easy to observe, and transgenic mice were generated that express AhR-CA in keratinocytes under the regulation of keratin 14 promoter (11). These transgenic mice were normal at birth, but severe skin lesions with itching developed postnatally. The skin lesions were accompanied by inflammation and immunological imbalance and resembled typical atopic dermatitis. This result clearly shows that constitutive activation of the AhR pathway is sufficient to trigger the inflammatory skin lesions and suggests the involvement of AhR-mediated transcription in the inflammatory diseases after exposure to occupational and environmental xenobiotics.

MATERIALS AND METHODS

Generating transgenic mice expressing AhR-CA. Male and female BDF_1 mice were purchased from CLEA Japan (Tokyo) to obtain fertilized eggs for DNA microinjection. AhR-CA transgenic mice, generated as described below, were maintained in the mixed background of C57BL/6 and DBA/2. AhR-CA transgenic mice and their wild-type littermates were housed in plastic cages in an air-conditioned room at a temperature of 24 ± 1°C, a humidity of 55% ± 5%, and a 12-h light-dark cycle.

For the construction of the pK14-AhR-CA-HA transgene, we first made a cDNA fragment encoding the constitutive active form of mouse AhR (AhR-CA) with a hemagglutinin (HA) tag sequence at its 3′ end (pBSK-AhR-CA-HA). The ligand-binding domain (277 to 418 amino acids [aa]) of AhR was deleted to generate AhR-CA. In brief, we generated AhR-CA cDNA by ligating the PCR-amplified fragments that encode the N-terminal (1 to 276 aa) and C-terminal (419 to 805 aa) halves of the AhR with an internal linker sequence (GGGGGA TCGGG), and the resultant cDNA fragment was subcloned into pBluescript II SK(+) vector (pBSK-mAhR-CA). An HA tag sequence was added to the 3′ end to make pBSK-AhR-CA-HA. To construct pK14-AhR-CA-HA, the blunt-ended HindIII/XbaI fragment of the HA-tagged AhR-CA cDNA was subcloned into the blunt-ended BamHI and XbaI sites of a human K14 promoter/enhancer cassette plasmid (a generous gift from E. Fuchs [11]). The construct was linearized, purified, and injected into fertilized oocytes. The transgenic mice were identified by the PCR (ca. 30 to 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) with a primer set: 5′-AAC TCT CTG TTC TTA GGC TCA GCG TC-3′ and 5′-ATA CGC TCT GAT GGA TGA CAT CAG ACT-3′. Fluorescence in situ hybridization (FISH) analysis was performed with the splenocytes prepared from Line 234 female mice and the pK14-AhR-CA-HA transgene as a probe through the standard procedure by Trans Animex Co., Ltd. (Hokkaido, Japan).

Immunoblotting analysis. Dorsal skins were homogenized in buffer containing 20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% protease inhibitor, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Homogenized samples were mixed with an equal volume of gel loading buffer (100 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and then sonicated and boiled at 100°C for 3 min. Soluble fractions were collected as supernatants after centrifugation. An aliquot (40 μg) of each sample was separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis and transferred onto Immobilon-P Transfer Membrane (Millipore Corp., Bedford, MA). The membrane was incubated in anti-HA antibody (sc-805; Santa Cruz Biotechnology, CA). The signal was detected using ECL and a Western blotting detection system (Amersham Pharmacia Biotech).

RNA blotting analysis. Total RNA was prepared from dorsal skin by using Isogen (Nippon Gene, Tokyo) according to the manufacturer's instructions. Total RNA (15 μg) was separated by electrophoresis on a 1% formaldehyde-agarose gel and subsequently transferred onto Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was probed with ^{32}P-labeled CYP1A1 cDNA fragment.

Histological and immunohistochemical analyses. Skins were fixed in 3.7% formaldehyde, embedded in paraffin, and sectioned. For histological analysis, the samples were stained with hematoxylin and eosin. Alcian blue staining was used for detecting mast cell granules. For the detection of CYP1A1, samples were allowed to react with anti-CYP1A1 antibody (sc-9828; Santa Cruz Biotechnology) overnight. Anti-CYP1A1 antibody binding was detected with horseradish peroxidase-conjugated anti-goat IgG antibody and diaminobenzidene was used as a chromogen.

Measurement of grooming duration and scratching frequency. Individual mice were kept in a cage in a calm state and monitored by video camera for 30 min. Grooming duration and scratching frequency were measured over a randomly selected 10-min period. Grooming of any part of the body using the forepaw or mouth was taken as a grooming episode, and a tally was made of the duration of each episode within the 10-min period. A series of scratching behavior using the hind paw was taken as one scratching episode, and the frequency of the episodes were counted for 10 min. Three mice were examined independently at 6 weeks of age.

cDNA microarray analysis. Ten-day-old AhR-CA mice of Line 239 (two males and three females) and their wild-type littermates (two males and two females) were sacrificed, and total RNAs were isolated from their back skin. The isolated RNAs were further purified by RNeasy RNA isolation kit (QIAGEN). Purified RNA was used for preparing labeled cRNA that was hybridized to a mouse expression array 430A gene chip (Affymetrix, Santa Clara, CA). Experimental procedures for GeneChip were performed according to the Affymetrix GeneChip expression analysis technical manual. The results were deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (GEO accession number GSE2955).

cDNA synthesis and reverse transcription-PCR (RT-PCR). Total RNA (3 μg) was reverse transcribed by Superscript II (Invitrogen) with 150 ng of random hexamer at 42°C. Each cDNA was amplified by PCR using ExTaq (TaKaRa Shuzo, Tokyo, Japan). The primers used were 5′-AGT GCA GAC AGT CCA CAG CA-3′ and 5′-TGC TCA GAG TAG TGA CCG AAC G-3′ for CYP1B1, 5′-TCA GTT CCC ATT GCA GTG G-3′ and 5′-TGG AAT GGA CTT GCC C-3′ for NQO1, 5′-ACA CGG TGC TGG ACG AAT C-3′ and 5′-ACG TAC GCC CAG TGA A-3′ for ADH, 5′-TTA CCA TGG GAG CTG AG-3′ and 5′-CCA GAG CGA AGC CAT TGA G-3′ for AhRR, 5′-ACC CGG ACC CAA AAC TTA G-3′ and 5′-TGT TTG CCA GCA GTG ATC-3′ for keratin 1, 5′-TTG AGC AGT ACA TCA GCA A-3′ and 5′-GGA TCA TGC GGT TGA-3′ for keratin 6, 5′-TCA TTA TTG CCA CCC AGG A-3′ and 5′-TCT CCA GGC CCT GGA A-3′ for keratin 16, 5′-TTC CTT GCT TTG GCA TG-3′ and 5′-CAG TTC TGC TTT GGA TC-3′ for CCL20, 5′-CAA CTT TGG CCG ACT TC-3′ and 5′-GAGT GAA CAT TAC AGA TTT ATC CCC-3′ for IL-18, 5′-CTA TCG TGC TCG AAT GAA CAC-3′ and 5′-GCC AAC AGG AAG CTG AGA GT-3′ for HO-1, and 5′-CGA GCA CAG CTT CTT T-3′ and 5′-AGG TAG TCT GTC AGG T-3′ for β-actin.

Measurement of serological parameters. Thirteen AhR-CA mice and twelve wild-type littermates aged 7 to 14 weeks were examined. Blood was drawn from retro-orbital vessels. IgE, IgG1, IgG2a, IgG3, and IgM were quantified by using enzyme-linked immunosorbent assay (ELISA). Cytokine production from spleen T cells stimulated with anti-CD3 monoclonal antibody. We treated 48-well flat-bottom microplates with 2 μg of anti-CD3 antibody (145-2C11; BD Pharmingen) and 0.5 μg of anti-CD28 antibody (PV-1; SBA). Splenocytes were prepared from seven AhR-CA mice and eight wild-type littermates aged from 7 to 14 weeks. A total of 5×10^6 cells were seeded per well and cultured with 1 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum for 24 h. The culture supernatants were then collected and interleukin-2 (IL-2), IL-4, IL-5, IL-12, and gamma interferon (IFN-γ) were quantified with ELISA.

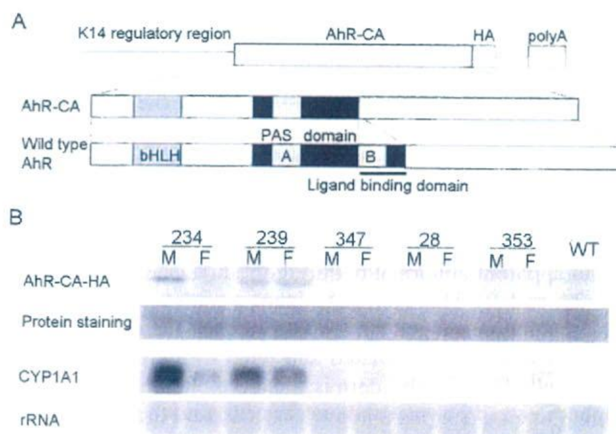


FIG. 1. Generation of AhR-CA transgenic mice. (A) Structure of the transgene. Basic helix-loop-helix (bHLH) and PAS domains are indicated as shaded and dotted boxes, respectively. The PAS-B domain serving as a ligand-binding domain (indicated by a bar) was deleted to generate the AhR-CA molecule. An HA tag was added at the C terminus of the protein. (B) Expression of the transgene correlates with the CYP1A1 mRNA level. HA-tagged AhR-CA was detected by immunoblot analysis with anti-HA antibody. CYP1A1 mRNA was detected by RNA blot analysis. The corresponding line numbers are shown at the top. M, male; F, female; WT, wild type.

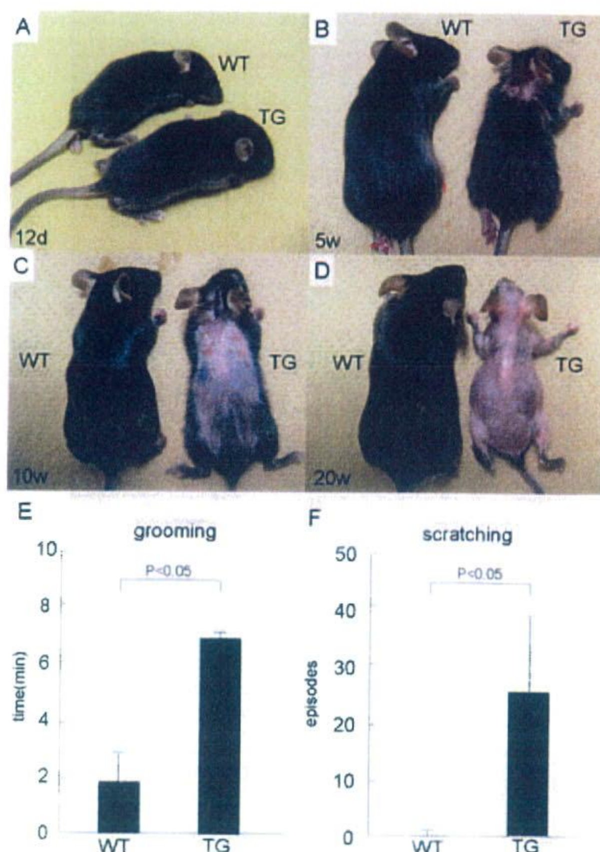


FIG. 2. Macroscopic observation of Line 239 mice. (A) Twelfth day after birth. Note that skin lesions are not apparent. (B) Fifth week. An eczematous change emerges on the back skin. (C) Tenth week. Erosive dermatitis with crusts is observed. (D) Twentieth week. Note that re-epithelialization follows, and the fur coat is completely lost. (E and F) Observation of behaviors related to skin itching. The total duration of grooming behavior within 10 min was measured for three independent mice, and the averages and standard deviations are shown in panel E. The frequency of scratching episodes within 10 min was counted for three independent mice, and the averages and standard deviations are shown in panel F. The differences between the AhR-CA mice (TG) and the wild-type control mice (WT) are statistically significant ($P < 0.05$).

RESULTS

Generation of transgenic mouse lines expressing AhR-CA in keratinocytes and integration of transgene into X chromosome in one of the lines. In order to explore the possibility that activation of the AhR pathway can elicit inflammatory response in vivo, we generated transgenic lines of mice that express AhR-CA in keratinocytes under the regulation of the keratin 14 (K14) promoter (Fig. 1A). Among several lines of mice generated, two (Lines 234 and 239) were found to express the AhR-CA transgene at high levels (Fig. 1B). Since the expression of AhR-CA in female Line 234 mice was consistently weaker than that in male mice of Line 234, we mapped the chromosomal localization of the transgene by using FISH analysis. The result unveiled that the transgene was integrated into the X chromosome in Line 234 (data not shown). Thus, we suspected that the transgene was subjected to random inactivation of the X chromosome (i.e., lionization [29]) and that the female skin was composed of two kinds of keratinocytes, the cells expressing the transgene and the cells whose transgenes were silenced, consequently resulting in the lowered expression of AhR-CA in female mice compared to male mice when the whole skin was examined.

Since the male mice of Line 234 died before weaning (data not shown), an essential gene on the X chromosome must have been disrupted by integration of the transgene. Line 234 mice, especially female mice, were found to provide strong evidence for a link between activation of the AhR pathway and the development of inflammatory lesions as will be described below. The abundance of AhR-CA showed a good correlation with the abundance of CYP1A1 mRNA, one of the target gene products of the AhR (Fig. 1B), demonstrating that AhR-mediated transcription is activated in the skin of the transgenic mice without any exogenous stimuli.

AhR-CA transgenic mice postnatally develop skin lesions with itching. Whereas Line 239 mice were normal at birth and healthy up to 12 days of age (Fig. 2A), they gradually developed erosive eczema in the back skin by 3 to 5 weeks after birth (Fig. 2B). Eventually, a massive loss of their fur coats occurred after the tenth week, even under specific-pathogen-free conditions (Fig. 2C and D). The whole skin over the body was subjected to the same process of skin lesion development. We noticed that both female and male mice of Line 239 frequently scratched their skin, which started around the time of weaning. Closer examination with the video tape recording revealed that both male and female transgenic mice of Line 239 performed grooming for longer periods of time and scratched much more frequently than their wild-type littermates (Fig. 2E and F). An important observation was that, although the transgene was already expressed in the embryos of the late fetal stage (data

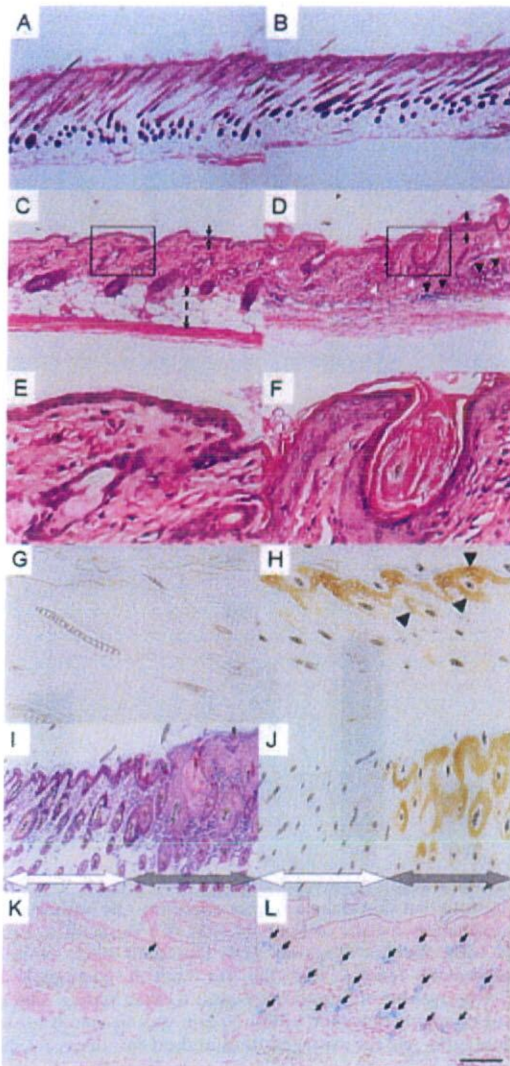


FIG. 3. Histological examination of the dorsal skin of AhR-CA mice. (A to F) Skin specimens from Line 239 AhR-CA transgenic mice (B, D, and F) and from wild-type mice (A, C, and E) were stained with hematoxylin and eosin. The samples were prepared at the age of 1 week (A and B) or 4 weeks (C to F). The black arrows (C and D) indicate the widths of the epidermal layers, and the dashed double arrowheads (C) indicate the width of the subcutaneous fat tissue. Black arrowheads (D) indicate cell infiltration, and white arrowheads (D) indicate dilated hair follicles filled with keratinized cell masses. Boxed areas in panels C and D are shown at a higher magnification in panels E and F, respectively. (G and H) Immunohistochemical detection of CYP1A1 expression. A 10-day-old Line 239 mouse (H) and its wild-type littermate (G) were examined. Intense brown signals can be seen in the epidermal and follicular keratinocytes (arrowheads) of the transgenic mouse, whereas no apparent signals can be detected in the control sample. (I and J) Serial sections prepared from the skin of a Line 234 adult female mouse. One was stained with hematoxylin and eosin (I), and the other was exposed to anti-CYP1A1 antibody (J). The left-hand halves of the micrographs (indicated by white double arrowheads) show the normal state of the skin, while the right-hand halves (indicated by gray double arrowheads) show inflammatory changes. (K and L) Alcian blue staining of the skin specimens from the 4-week-old mice of Line 239 (L) and wild-type littermate (K). Black arrows (K and L) indicate mast cells that have infiltrated the dermis. Scale bar: 40 μm (A and B), 16 μm (C, D, K, and L), 8 μm (G to J), 4 μm (E and F).

not shown), skin disorders accompanied by itching and scratching developed postnatally in Line 239 mice, suggesting that postnatal epidermal development and/or mechanical stimuli may be required in addition for completion of the skin lesion pathogenesis.

Epidermal and dermal pathological changes observed in AhR-CA transgenic mice. To examine the skin of symptomatic Line 239 mice in more detail, histological analysis was performed. Consistent with the macroscopic observation, there was no apparent abnormality during the first week after birth (Fig. 3A and B). However, prominent acanthosis and hyperkeratinization developed by 4 weeks of age (Fig. 3D and F); most hair follicles were dilated and filled with layers of keratinized cells (6, 37). The dermis was severely infiltrated with lymphocytes and polymorphonuclear cells, and the subcutaneous fat tissue had mostly disappeared (Fig. 3D and F).

Although the macroscopic skin lesions observed in Line 234 were milder than in Line 239, both lines shared similar histopathological changes. Male Line 234 mice, with the highest transgene expression (see Fig. 1B), developed the pathological state by 2 weeks of age, which was slightly earlier than the onset of Line 239 mice (data not shown). Further exacerbation could not be observed in male Line 234 mice since the mice die before weaning due to a probable insertion mutagenesis or disruption of an essential gene on the X chromosome by the transgene. Intriguingly, adult female Line 234 mice displayed chimeric patterns in their skin lesions, which made the dermatitis macroscopically less apparent. Both normal and inflammatory parts resided side by side (Fig. 3I), and this pattern was quite consistent with that of the target gene expression (Fig. 3J). This observation may be best explained by lionization of the transgene integrated into X chromosome. We can assume that, in the normal part of skin, the AhR-CA transgene was inactivated due to the lionization of the transgene-harboring X chromosome, whereas, in the inflammatory part of the skin, the expression of the transgene persisted due to the transgene-harboring X chromosome escaping from the lionization. None of our low AhR-CA transgene expressor lines showed any such skin abnormalities (data not shown). These results thus indicate that the severity of the skin disorders correlates well with the expression levels of the AhR-CA transgene.

We then monitored the activity of AhR-CA protein in the skin through detecting expression of its target gene, CYP1A1. When CYP1A1 expression in the transgenic mice was examined immunohistochemically, intense signals were detected specifically in epidermal keratinocytes, an observation consistent in Line 239 mice (Fig. 3H) and male Line 234 mice at 10 days after birth (data not shown). As expected, female Line 234 mice displayed a chimeric staining pattern (Fig. 3J). A patch of epidermis with intense CYP1A1 expression displayed acanthotic proliferation and hyperkeratinization, and the underlying dermis was infiltrated with inflammatory cells (Fig. 3I and J). However, such skin alterations were not observed in the portions without a detectable CYP1A1 expression. These results clearly indicate that the AhR pathway is activated specifically in keratinocytes and that this keratinocyte-specific AhR activation causes the observed inflammatory skin disorders.

Alcian blue staining of the skin sections revealed that a large number of mast cells had accumulated in the dermis in Line 239 (Fig. 3L). This was also evident in Line 234 males at 10 days after

TABLE 1. Genes increased in the skin of AhR-CA mice

Gene category	Gene subcategory	Accession no.	Gene	Fold change
Detoxifying enzymes	Detoxifying enzymes and genes	NM009644	Aryl-hydrocarbon receptor repressor	16.6
		NM009992	Cytochrome P450, 1a1	44.2
		NM009994	Cytochrome P450, 1b1	4.0
		NM007825	Cytochrome P450, 7b1	3.7
		AV158882	NAD(P)H dehydrogenase, quinone 1	3.8
		NM010357	Glutathione *S*-transferase, alpha 4	8.2
		NM008184	Glutathione *S*-transferase, mu 6	2.0
		NM008161	Glutathione peroxidase 3	2.7
Interleukins, chemokines, and receptors	Interleukins and their receptors	AF000304	IL-4 receptor, alpha	15.1
		L20048	IL-2 receptor, gamma chain	8.0
		D13695	IL-1 beta	6.0
		NM008360	IL-18	2.2
	Chemokines	BC002073	Chemokine (C-C motif) ligand 6	18.5
		NM009141	Chemokine (C-X-C motif) ligand 5	22.2
		AF099052	Chemokine (C-C motif) ligand 20	14.9
		NM008176	Chemokine (C-X-C motif) ligand 1	8.9
		NM011888	Chemokine (C-C motif) ligand 19	8.4
		NM021443	Chemokine (C-C motif) ligand 8	6.3
Immunological proteins	Fc receptors	NM010185	Fc receptor, IgE, high-affinity I, Gamma polypeptide	23.9
		BM224327	Fc receptor, IgG, low-affinity IIb	6.8
	Antimicrobial peptide	NM019728	Defensin beta 4	38.3
		NM013756	Defensin beta 3	11.0
Structure proteins	Keratins	NM010669	Keratin 6b	35.1
		NM008470	Keratin 16	7.8
		NM008473	Keratin 1	6.1
Proteases and inhibitors	Proteases	NM008572	Mast cell protease 8	11.1
		NM008571	Mast cell protease 2	2.8
		NM008607	Matrix metalloproteinase 13	34.8
		AV375008	Matrix metalloproteinase 19	9.1
		NM013599	Matrix metalloproteinase 9	3.3

birth (data not shown) but less evident in Line 234 females. Since mast cells are considered to be a critical factor inducing skin itching, we can deduce that the skin lesions are intimately associated with the itching.

Inflammation-related genes are increased in AhR-CA mice. To further clarify the molecular basis of the skin lesions, we performed a DNA microarray analysis with skin RNA samples from 10-day-old Line 239 mice, an age representing initiation of the skin disorders (Table 1 and Fig. 4). This early stage was chosen because we were curious about how the skin lesions were initiated. Total RNAs from five Line 239 mice and four wild-type littermates were pooled as AhR-CA RNA and wild-type RNA, respectively, and these two samples were compared. Among 309 genes that were increased >4-fold in the transgenic mice compared to wild-type mice, 7% were involved in detoxification, while, surprisingly, nearly a quarter were inflammation-related genes, including interleukins/chemokines and their receptors, immunological proteins, and proteases and their inhibitors (Fig. 4A). We looked at genes with an expression either upregulated or downregulated by at least twofold and found that the ratios of the upregulated genes were higher in the categories of the inflammation-related genes (Fig. 4B).

We then selected representative genes from the microarray analysis and examined their expression levels by RT-PCR analysis with 10-day-old skin RNAs (Fig. 4C). All of the genes, except for heme oxygenase-1 (HO-1), were consistently increased in Line 239 and male Line 234 mice and slightly increased in female Line 234 mice. For instance, the detoxifying enzymes CYP1B1, NAD(P)H:quinone oxidoreductase 1, alcohol dehydrogenase, and AhR repressor were higher in the transgenic mice compared to wild-type levels. As for the inflammation-related genes, keratins 1, 6, and 16 (K1, K6, and K16), CCL20, and IL-18 were dramatically increased in Line 239 and male Line 234 mice, reflecting the proinflammatory state of the skin of these transgenic mice.

We found that IL-18 and CCL20 were also highly expressed in the fetal skin of AhR-CA mice soon after the start of transgene expression (data not shown). In addition, considering that the upstream regions of the *IL-18* and *CCL20* genes contain multiple AhR binding sites (xenobiotic response elements [XRE]), *IL-18* and *CCL20* could be the primary genes activated by AhR-CA in keratinocytes to trigger the inflammatory responses.

An intriguing observation was that, HO-1, considered a sensitive marker of the oxidative stress generated by exposure to PAHs (20), was not induced in the AhR-CA transgenic mice, suggesting that contribution of reactive oxygen species to the development of the skin lesions might be marginal.

The cell-mediated immune response is predominant in AhR-CA transgenic mice. We then examined characteristics of fully developed skin inflammation observed in AhR-CA trans-

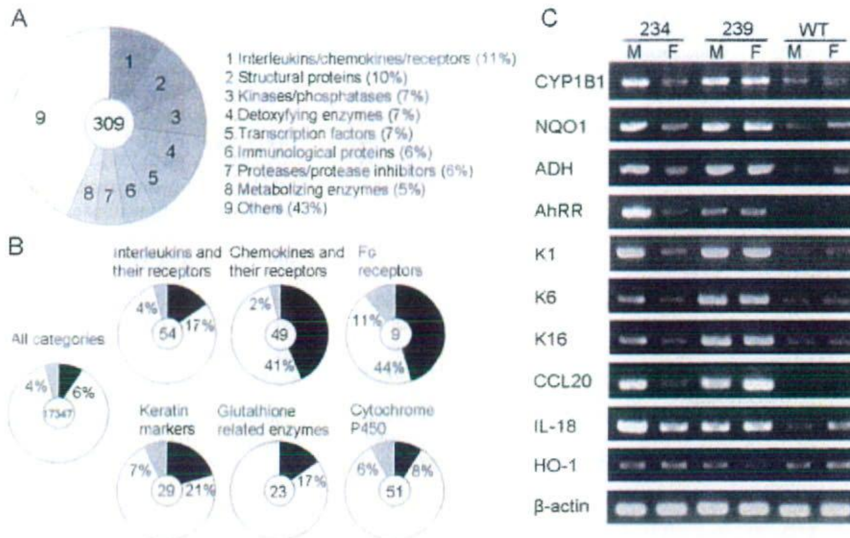


FIG. 4. Altered gene expression profiles in the skin of AhR-CA mice. (A) A total of 309 genes increased by >4-fold are categorized into nine groups. The percentage of each category is shown. (B) The ratios of the genes increased (black areas) or deceased (gray areas) by >2-fold are shown within each gene subcategory. The number written in the center of each circle graph indicates the number of genes included in that subcategory. (C) The expression levels of representative genes were examined by semiquantitative RT-PCR. cDNAs were synthesized from the skin RNAs of 10-day-old AhR-CA and wild-type mice. The corresponding line numbers are shown at the top. M, male; F, female; WT, wild type.

genic mice using adult Line 239 mice. Since local inflammation is often accompanied by systemic immune imbalance, we assessed whether the AhR-CA transgenic mice display any signs of disturbance of the immune system, particularly, imbalance between Th1 and Th2 cells. Differentiated $CD4^+$ lymphocytes (helper T cell) residing in spleen are divided into two populations, Th1 cells and Th2 cells, based on the potential ability of cytokine production. IFN-γ and IL-2 are the cytokines preferentially produced by Th1 cells, and IL-4 and IL-5 are the ones produced by Th2 cells when they are stimulated (e.g., by anti-CD3 antibody).

To examine the balance between Th1 and Th2 cell populations, we first measured the serum concentration of immunoglobulin and found that the concentrations of both IgG1 and IgE in the sera of Line 239 mice were markedly elevated compared to those in the wild-type littermates (Fig. 5A). Since the production of IgG1 and IgE is promoted by the cytokines derived from Th2 lymphocytes, it was strongly suggested that a Th2 cell-mediated immune response was predominant in symptomatic mice and that systemic immune balance was secondarily affected by epidermal proinflammatory responses.

To further address this point, we then isolated and cultured splenocytes of adult Line 239 mice and measured the cytokine production. Consistent with our current results, splenocytes from the symptomatic mice produced much more IL-4 and IL-5, which were secreted from Th2 cells, in response to immobilized anti-CD3 antibody (Fig. 5B). On the other hand, the production of IL-2 and IFN-γ (secreted by Th1 cells) was lower in the Line 239 mice than in wild-type mice. Taken together, we concluded that overexpression of AhR-CA gives rise to high serum IgE and IgG1 levels and a dominant Th2 response. It is noteworthy that these immune responses are often common background phenotypes accompanying atopic dermatitis (19) and a frequent consequence of exposure to environmental xenobiotics, including diesel exhaust particles (8).

DISCUSSION

We demonstrated here that keratinocyte-targeted overexpression of the AhR-CA protein represents an effective way of generating skin lesions that mimic atopic dermatitis and PAH contact hypersensitivity (8, 19). We successfully generated inflammatory skin lesions with no systemic or topical application of any exogenous chemicals, but by purely activating AhR-mediated transcription in epidermal keratinocytes. Our microarray result showed that many inflammation-related genes were actually upregulated, which is a good reflection of our unique strategy. These results clearly indicate that activation of the AhR signaling pathway itself is sufficient to initiate the inflammatory disorders.

The K14 promoter was reported to be active in the whole layers of epidermal keratinocytes, including follicular keratinocytes, when examined in transgenic mice (11). This expression pattern was consistent with that of CYP1A1 examined here (see Fig. 3H) and similar to that of endogenous AhR in skin (J. Mimura and Y. Fujii-Kuriyama, unpublished observations). Thus, AhR-CA expression driven by the K14 promoter was not merely ectopic but rather mimicked endogenous AhR distribution, allowing an interpretation that AhR-CA mice reflect a condition where AhR ligands are applied to the skin without operating AhR-independent action.

Transgenic mice expressing AhR-CA under the regulation of the mouse immunoglobulin heavy-chain enhancer showed an elevated risk in the spontaneous development of stomach tumors (2) and chemical hepatocarcinogenesis (23). When AhR-CA was expressed in mice under the regulation of T

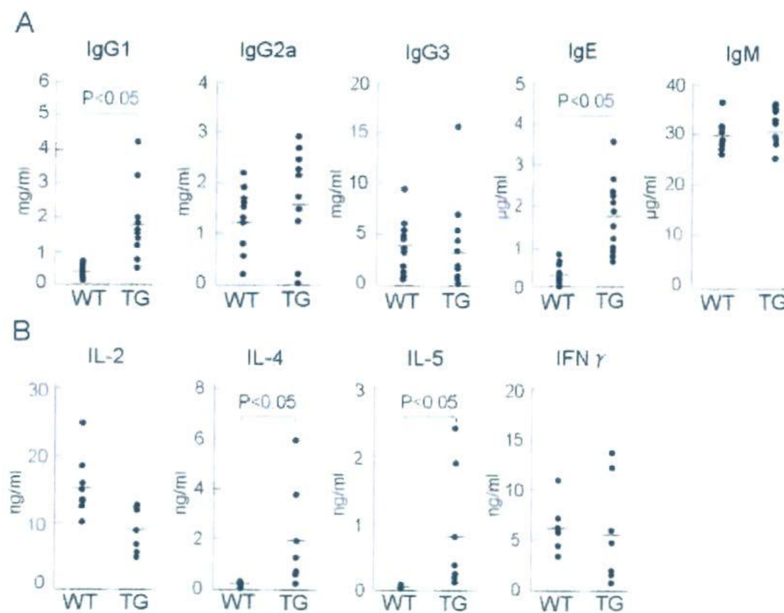


FIG. 5. Spontaneous deviation of splenic lymphocytes into Th2 cells. (A) High serum levels of IgE and IgG1 in AhR-CA mice. Sera from the AhR-CA mice ($n = 13$) and their wild-type littermates ($n = 12$) aged from 7 to 14 weeks were sampled. The serum levels of various types of immunoglobulins (IgG2a, IgG3, IgM, IgE, and IgG1) were measured by ELISA. Differences in the levels of IgE and IgG1 are statistically significant between the transgenic mice and controls ($P < 0.05$). (B) Increased production of IL-4 and IL-5 from the anti-CD3 stimulated splenic lymphocytes. Splenic lymphocytes were prepared from the AhR-CA mice ($n = 7$) and their wild-type littermates ($n = 8$) aged from 7 to 14 weeks and then stimulated with anti-CD3 antibody. The cytokine concentrations (IL-4, IL-5, IFN-γ, and IL-2) in each supernatant were determined by ELISA. Differences in the levels of IL-4 and IL-5 are statistically significant between the transgenic mice and their controls ($P < 0.05$).

cell-specific CD2 promoter, the number of thymocytes was decreased and immunization-induced T-cell or B-cell expansion was suppressed (26). Importantly, none of these reports described the development of inflammation in any tissues. In contrast, the present results provide convincing evidence that the local activation of the AhR is critical for provoking the inflammatory responses in the tissues involved. The patchy skin lesions observed in Line 234 females nicely support this contention. Thus, avoidance of local exposure to PAHs should be effective in preventing inflammatory disorders mediated by PAHs.

The development of inflammatory skin diseases has been suggested to be the result of interactions between the immune system and skin-derived molecules (18, 25). Indeed, it was previously suggested that topically applied PAHs exert a systemic influence on immune organs (16). Although the precise mechanism remains to be elucidated, we speculate that this either occurs as a direct effect of PAHs on the immune cells after percutaneous absorption or by the secondary activation of immune organs following proinflammatory responses of the skin. The AhR-CA transgenic mice displayed skin lesions accompanied by the systemic immune imbalance shifted toward the Th2 cell predominance, which could be the secondary effect of the epidermal proinflammatory responses mediated by AhR-CA protein. In addition, the direct activation of the AhR pathway in the immune organs also seemed to be responsible for the full completion of the pathological state of the transgenic mice. Consistent with the previous observation of K14 promoter activity (9), we found the additional expression of AhR-CA in the thymus (data not shown), which may have contributed to the altered immune system together with cytokines and chemokines derived from the skin lesions.

There is an interesting discrepancy between the onset of apparent inflammation in histology and that of inflammation-related gene induction. The former coincides with the postnatal period around the weaning age, and the latter starts within the late fetal stage in utero. Although the precise reason why the skin lesions develop only after birth remains unknown, we speculate that maturation of the immune systems may be required before the primary triggering signals originated from the keratinocytes come into effect for the progression of the inflammatory process. The development and maturation of skin itself might also be required.

Scratching behavior seems to exacerbate the progression of the skin lesions, ending in the massive loss of fur coats. Although prominent acanthosis and hyperkeratinization with dermal infiltration of lymphocytes and polymorphonuclear cells were commonly observed in both Line 239 and Line 234 mice, the severe scratching and dramatic skin lesions were prominently observed in Line 239 mice but not in Line 234 mice. The male mice of Line 234 die around the weaning, so they do not live until they were able to scratch their skin. Female mice of Line 234 scratch their skin only mildly; probably the itching may not be so strong because of the spotted inflammation in their skin. Hence, the further evolvement of the skin lesions seems to correlate well with the presence of scratching behavior.

One important question remained is which genes are responsible for triggering the skin inflammation in response to the constitutively active AhR. Among the various genes in-

duced in the transgenic skin, IL-18 and CCL20 are good candidates for triggering the inflammatory responses. IL-18 is known to induce inflammatory skin diseases similar to atopic dermatitis when expressed in mice through the K14 promoter (17). Similarly, if secreted from keratinocytes, CCL20 would be able to trigger skin inflammation by directing the migration of inflammatory cells (34). Indeed, there has been a strong suggestion of a correlation between CCL20 and atopic dermatitis (24). Multiple XRE found in the upstream regions of the *IL-18* and *CCL20* genes imply that these genomic regions are responsive to the activated AhR. However, other proinflammatory genes that do not contain an XRE in the proximal promoter region may also have contributed, since recent studies revealed the XRE-independent AhR function as a coactivator for other transcription factors (27). Further investigation is necessary for elucidation of the primary target genes that are activated by AhR-CA and trigger the inflammatory responses.

Intriguingly, the phenotype of the skin lesions, with remarkable cell infiltration and itching, resembles the contact dermatitis triggered by other PAHs (5) more than the chloracne triggered by dioxins (6, 37), implying the operation of distinct mechanisms underlying the two pathological conditions. A previous report showed that binding sequences are different between dioxin-liganded AhR and PAH-liganded AhR (21a), suggesting that a profile of gene activation by AhR after dioxin exposure could be different from that by AhR after PAH exposure. We surmise that a profile of gene activation by AhR-CA might be more similar to that by PAH-liganded AhR. Tissue delivery and distribution of dioxins and PAHs might be another factor to generate the phenotypic difference. It seems likely that AhR activation triggered in PAH contact dermatitis predominantly occurs in keratinocytes, as seen in AhR-CA mice we generated in the present study.

The present study demonstrates for the first time the primary contribution of AhR-mediated transcriptional activation to the development of inflammatory diseases. Since no exogenous chemical was required to trigger the inflammatory responses in this model system, activation of the downstream genes under AhR regulation is most likely to be responsible. Our findings suggest that the induction of AhR target genes is one of the central mechanisms of PAH-mediated inflammatory diseases. Based on these findings, the possibility emerges that blocking AhR signals may help to relieve the allergic symptoms, since xenobiotic exposure exacerbates allergic diseases through the AhR pathway.

ACKNOWLEDGMENTS

We thank Yuko Kikuchi, Naomi Kaneko, and Reiko Kawai for assistance. We also thank Yoichi Matsuda and Kazuhiko Yamada at Trans Animex for FISH analysis, Tania O'Connor for help with editing the manuscript, and Shogo Yamamoto for help in deposition of the microarray data.

This study was supported by grants from ERATO-JST (M.Y.); the Ministry of Education, Science, Sports, and Culture (H.M. and M.Y.); the Ministry of Health, Labor, and Welfare (M.Y.); the Atherosclerosis Foundation (M.Y.); SORST-JST (Y.F.-K. and H.M.); the Yamanouchi Foundation for Research on Metabolic Disorders (H.M.); the Uehara Memorial Foundation (H.M.); and the Special Coordination Fund for Promoting Science and Technology (H.M.). F.K. is a JSPS Research Fellow.

REFERENCES

1. **Anderson, C., A. Hebr, R. Robbins, R. Hasan, M. Athar, H. Mukhtar, and C. A. Elmets.** 1995. Metabolic requirements for induction of contact hypersensitivity to immunotoxic polyaromatic hydrocarbons. J. Immunol. **155:** 3530–3537.
2. **Andersson, P., J. McGuire, C. Rubio, K. Gradin, M. L. Whitelaw, S. Pettersson, A. Hanberg, and L. Poellinger.** 2002. A constitutively active dioxin/aryl hydrocarbon receptor induces stomach tumors. Proc. Natl. Acad. Sci. USA **99:**9990–9995.
3. **Arruda, L. K., D. Sole, C. E. Baena-Cagnani, and C. K. Naspitz.** 2005. Risk factors for asthma and atopy. Curr. Opin. Allergy Clin. Immunol. **5:**153–159.
4. **Bonvallot, V., A. Baeza-Squiban, A. Baulig, S. Brulant, S. Boland, F. Muzeau, R. Barouki, and F. Marano.** 2001. Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. Am. J. Respir. Cell. Mol. Biol. **25:**515–521.
5. **Casale, G. P., Z. Cheng, J. Liu, E. L. Cavalieri, and M. Singhal.** 2000. Profiles of cytokine mRNAs in the skin and lymph nodes of SENCAR mice treated epicutaneously with dibenzo[a,l]pyrene or dimethylbenz[*a*]anthracene reveal a direct correlation between carcinogen-induced contact hypersensitivity and epidermal hyperplasia. Mol. Carcinog. **27:**125–140.
6. **Coenraads, P. J., A. Brouwer, K. Olie, and N. Tang.** 1994. Chloracne: some recent issues. Dermatol. Clin. **12:**569–576.
7. **Davila, D. P. D. R. Davis, K. Campbell, J. C. Cambier, L. A. Zigmond, and S. W. Burchiel.** 1995. Role of alterations in Ca^{2+}-associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. J. Toxicol. Environ. Health **45:**101–126.
8. **Diaz-Sanchez, D.** 1997. The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. Allergy **52:**52–56.
9. **Frazer, I. H., G. J. P. Fernando, N. Fowler, G. R. Leggatt, P. F. Lambert, A. Liem, K. Malcolm, and R. W. Tindle.** 1998. Split tolerance to a viral antigen expressed in thymic epithelium and keratinocytes. Eur. J. Immunol. **28:** 2791–2800.
10. **Fujimaki, H., K. Nohara, T. Kobayashi, K. Suzuki, K. Eguchi-Kasai, S. Tsukumo, M. Kijima, and C. Tohyama.** 2002. Effect of a single oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on immune function in male NC/Nga mice. Toxicol. Sci. **66:**117–124.
11. **Guo, L., Q. C. Yu, and E. Fuchs.** 1993. Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice. EMBO J. **12:**973–986.
12. **Hankinson, O.** 1995. The aryl hydrocarbon receptor complex. Annu. Rev. Pharmacol. Toxicol. **35:**307–340.
13. **Heo, Y., A. Saxon, and O. Hankinson.** 2001. Effect of diesel exhaust particles and their components on the allergen-specific IgE and IgG1 response in mice. Toxicology **159:**143–158.
14. **Karras, J. G., D. H. Conrad, and M. P. Holsapple.** 1995. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on interleukin-4-mediated mechanisms of immunity. Toxicol. Lett. **75:**225–233.
15. **Kepley, C. L., F. T. Lauer, J. M. Oliver, and S. W. Burchiel.** 2003. Environmental polycyclic aromatic hydrocarbons, benzo(a)pyrene(BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils. Clin. Immunol. **107:**10–19.
16. **Klemme, J. C., H. Mukhtar, and C. A. Elmets.** 1987. Induction of contact hypersensitivity to dimethylbenz(a)anthracene and benzo(a)pyrene in C3H/HeN mice. Cancer Res. **47:**6074–6078.
17. **Konishi, H., H. Tsutsui, T. Murakami, S. Yumikura-Futatsugi, K. Yamanaka, M. Tanaka, Y. Iwakura, N. Suzuki, K. Takeda, S. Akira, K. Nakanishi, and H. Mizutani.** 2002. IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions. Proc. Natl. Acad. Sci. USA **99:**11340–11345.
18. **Leung, D. Y.** 1999. Pathogenesis of atopic dermatitis. J. Allergy Clin. Immunol. **104:**99–108.
19. **Leung, D. Y., and T. Bieber.** 2003. Atopic dermatitis. Lancet **361:**151–160.
20. **Li, N., A. C. Sioutas, Cho, D. Schmitz, C. Misra, J. Sempf, M. Wang, T. Oberley, J. Froines, and A. Nel.** 2003. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. Environ. Health Perspect. **111:** 455–460.
21. **Mastrangelo, G., C. Veller Fornasa, S. Pavanello, G. Mercer, M. Lazzaro, Milan, E. G. Fadda, U. Fedeli, and E. Clonfero.** 2003. Polyaromatic hydrocarbons administered in humans by dermal route increase total IgE. Int. J. Immunopathol. Pharmacol. **16:**145–150.

21a. **Matikainen, T., G. I. Perez, A. Jurisicova, J. K. Pru, J. J. Schlezinger, H. Y. Ryu, J. Laine, T. Sakai, S. J. Korsmeyer, R. F. Casper, D. H. Sherr, and J. L. Tilly.** 2001. Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. Nat. Genet. **28:**355–360.

22. **Mimura, J., K. Yamashita, K. Nakamura, M. Morita, T. N. Takagi, K. Nakao, M. Ema, K. Sogawa, M. Yasuda, M. Katsuki, and Y. Fujii-Kuriyama.**

1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells **2**:645–654.

23. **Moennikes, O., S. Loeppen, A. Buchmann, P. Andersson, C. Ittrich, L. Poellinger, and Schwarz. M.** 2004. A constitutively active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice. Cancer Res. **64:** 4707–4710.
24. **Nakayama, T., R. Fujisawa, H. Yamada, T. Horikawa, H. Kawasaki, K. Hieshima, D. Izawa, S. Fujiie, T. Tezuka, and O. Yoshie.** 2001. Inducible expression of a CC chemokine liver- and activation-regulated chemokine (LARC)/macrophage inflammatory protein (MIP)-3α/CCL20 by epidermal keratinocytes and its role in atopic dermatitis. Int. Immunol. **13**:95–103.
25. **Nickoloff, B. J.** 1999. Skin innate immune system in psoriasis: friend or foe. J. Clin. Investig. **104**:1161–1164.
26. **Nohara, X. K., Pan, S. Tsukumo, A. Hida, T. Ito, H. Nagai, K. Inouye, H. Motohashi, M. Yamamoto, Y. Fujii-Kuriyama, and C. Tohyama.** 2005. Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses the immunization-induced increase in splenocytes. J. Immunol. **174**:2770–2777.
27. **Ohtake, F., K. Takeyama, T. Matsumoto, H. Kitagawa, Y. Yamamoto, K. Nohara, C. Tohyama, A. Krust, J. Mimura, P. Chambon, J. Yanagisawa, Y. Fujii-Kuriyama, and S. Kato.** 2003. Modulation of estrogen receptor signalling by association with the activated dioxin receptor. Nature **423**:545–550.
28. **Pei, X. H., Y. Nakanishi, H. Inoue, K. Takayama, F. Bai, and N. Hara.** 2002. Polycyclic aromatic hydrocarbons induce IL-8 expression through nuclear factor κB activation in A549 cell line. Cytokine **19**:236–241.
29. **Pereira, L. V., and L. R. Vasques.** 2000. X-chromosome inactivation: lessons from transgenic mice. Gene **255**:363–371.
30. **Rydzynski, K., and C. Palczynski.** 2004. Occupational allergy as a challenge to developing countries. Toxicology **198**:75–82.
31. **Saxon, A., and D. Diaz-Sanchez.** 2000. Diesel exhaust as a model xenobiotic in allergic inflammation. Immunopharmacology **48**:325–327.
32. **Sjogren, M., L. Ehrenberg, and U. Rannug.** 1996. Relevance of different biological assays in assessing initiating and promoting properties of polycyclic aromatic hydrocarbons with respect to carcinogenic potency. Mutat. Res. **358**:97–112.
33. **Takenaka, H., K. Zhang, D. Diaz-Sanchez, A. Tsien, and A. Saxon.** 1995. Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: direct effects on B-cell IgE production. J. Allergy Clin. Immunol. **95**:103–115.
34. **Tohyama, M., Y. Shirakara, K. Yamasaki, K. Sayama, and K. Hashimoto.** 2001. Differentiated keratinocytes are responsible for TNF-alpha regulated production of macrophage inflammatory protein 3α/CCL20, a potent chemokine for Langerhans cells. J. Dermatol. Sci. **27**:130–139.
35. **Walker, D. B., W. C. Williams, C. B. Copeland, and R. J. Smialowicz.** 2004. Persistent suppression of contact hypersensitivity, and altered T-cell parameters in F344 rats exposed perinatally to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicology **197**:57–66.
36. **Wu, M. T., C. H. Pan, T. N. Wu, Y. L. Huang, C. Y. Chen, L. H. Huang, and C. K. Ho.** 2003. Immunological findings in a group of coke-oven workers exposed to polycyclic aromatic hydrocarbons. J. Occup. Environ. Med. **45:** 1034–1039.
37. **Yamamoto, O., and Y. Tokura.** 2003. Photocontact dermatitis and chloracne: two major occupational and environmental skin diseases induced by different actions of halogenated chemicals. J. Dermatol. Sci. **32**:85–94.

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

BBRC

Biochemical and Biophysical Research Communications 338 (2005) 311–317

www.elsevier.com/locate/ybbrc

Review

Molecular mechanisms of AhR functions in the regulation of *cytochrome P450* genes

Y. Fujii-Kuriyama *, J. Mimura

Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba 305-8577, Japan
Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Honcho 4-1-8, Kawaguchi 332-0012, Japan

Received 17 August 2005
Available online 30 August 2005

Abstract

AhR, a ligand-activated transcription factor, mediates xenobiotic signaling to enhance the expression of target genes, including drug-metabolizing cytochrome P450s. The recent development of several new techniques, including chromatin immunoprecipitation and RNA interference, has expanded and deepened our knowledge of AhR function in the xenobiotic signal transduction. In this review, we briefly summarize our current understanding of the activation and inactivation of AhR activities and discuss the future directions of AhR research.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Aryl hydrocarbon receptor; Cytochrome P450; Transcription factor; Coactivator; Gene regulation

Cytochrome P450 (CYP) is a superfamily of hemoproteins, composed of more than 3000 molecules and distributed across species ranging from bacteria to vertebrates. These proteins catalyze the monooxygenation of various endogenous and exogenous substrates [1,2]. Superfamily members are classified according to the similarity of their primary structures; members of families 1, 2, 3, and 4 are mainly involved in the metabolism of exogenous chemicals, including drugs, food additives, and environmental pollutants. CYPs are typically inducible; specific forms of CYPs are induced in response to the administration of certain chemicals [3–5].

Recently, the molecular mechanisms governing the inducible expression of CYPs have been successfully elucidated, including the inducers, *cis*-acting DNA elements, cognate transcription factors, and coactivators. The inducible expression of the CYP1 family is regulated by a heterodimer of the aryl hydrocarbon receptor (AhR or dioxin receptor) and the aryl hydrocarbon receptor nuclear translocator (Arnt), which contain a bHLH-PAS structural motif [5,6]. Expressions of the CYP2, 3, and 4 family members are controlled by other transcription factors (CAR, PXR, and PPAR) of the nuclear receptor (NR) superfamily, which have a characteristic zinc finger motif different from that in AhR [3–5].

The specific involvement of these receptor-type transcription factors in the induction of certain CYPs has been confirmed by gene-engineering technology, including gene-knockout methods [3,6–8]. The recent development of new techniques, including small interference RNA (siRNA) and chromatin immunoprecipitation (Chip) analyses, has greatly expanded our knowledge of the molecular mechanisms controlling the inducible expression of drug-metabolizing CYPs [9]. In this short review, we summarize the recent advances in the study of AhR activation and inducible expression of CYP1 and some other CYP families. The mechanisms of NR-related inducible expression of CYP2, 3, and 4 families are addressed in a number of excellent review articles [10–12].

Activation of AhR

Normally, AhR exists in a dormant state within the cytoplasm in association with a complex of HSP90, XAP2, and p23. Upon ligand binding, AhR in the complex is activated by a conformation change that exposes a

* Corresponding author. Fax: +81 29 853 7318.
E-mail address: ykfujii@tara.tsukuba.ac.jp (Y. Fujii-Kuriyama).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.162

nuclear localization signal(s) (NLS). The ligand-activated AhR in the complex translocates into the nucleus and forms a heterodimer with the closely related Arnt protein already present in the nucleus by dissociating from the complex [5,6].

Structure–activity relationship studies examining the binding activity of AhR ligands using a large number of halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) suggest that the presumed AhR binding pocket accepts planar ligands with maximal van der Waal's dimensions of $14 \times 12 \times 5$ Å [13,14] (Fig. 1). Some electronic and thermodynamic properties of ligands appear to be important for a high binding affinity, although formulation of the exact structure necessary has yet to be defined [14]. 2′,3′,7′,8′-Tetrachlorodibenzo-*p*-dioxin (TCDD) and indolo[2,3-*b*]carbazole (ICZ) are the most potent inducers of CYP1 expression. AhR, which is well conserved from invertebrates like *Caenorhabditis elegans* and *Drosophila melanogaster* to vertebrates [15], mediates the majority of pharmacological and toxicological effects on host animals elicited by those xenobiotics. This high degree of evolutionary conservation among species suggests that AhR plays an important physiological role in homeostasis and/or development. In support of this hypothesis, many endogenous compounds with chemical properties different from those of the known high affinity xenobiotic ligands, such as tryptamine and indole acetic acid [16], bilirubin and biliverdin [17], and lipoxin A4 [18], have been isolated as potential natural ligands of AhR (Fig. 1). These compounds have relatively low binding affinities for AhR in comparison to those of TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF), and ICZ. Of the tryptophan-derived natural AhR ligands like indirubin and indigo, 6-formylindolo[3,2-*b*]carbazole (FICZ), a tryptophan photoproduct, has a very high affinity for AhR, comparable to that of TCDD (Fig. 1). FICZ is formed in cell culture medium exposed to light in the presence of riboflavin [19]. Identification of all these compounds as active ligands for AhR was conducted by examining their ability to induce *CYP1A1* gene expression in cultured cells or by measuring their xenobiotic response element (XRE)-binding activity by gel mobility shift assay (GMSA). Although these chemicals have the potential to activate AhR activity, identification of a true physiological ligand for AhR would require to clarify how the activation of AhR by these naturally occurring ligands is associated with specific physiological functions.

Recently, a number of papers have reported that AhR is activated in the absence of obvious ligands in Hepa 1clc7 cells [20], human keratinocytes [21], 10T1/2 fibroblasts [22], and HaCaT cells [23] grown under specific culture conditions. When Hepa-1 cells and human keratinocytes were grown in suspension, CYP1A1 mRNA was induced without the addition of AhR ligands in an AhR-dependent manner at levels similar to that seen following TCDD treatment [20,21]. Suspension cultures of several C3H10T1/2 fibroblast clonal sub-lines that contain an integrated AhR-responsive reporter gene exhibited a time-course and levels of reporter activation and endogenous CYP1B1 induction that paralleled TCDD stimulation in confluent monolayer culture. Loss of cell–cell contacts at low culture densities also activated the expression of the

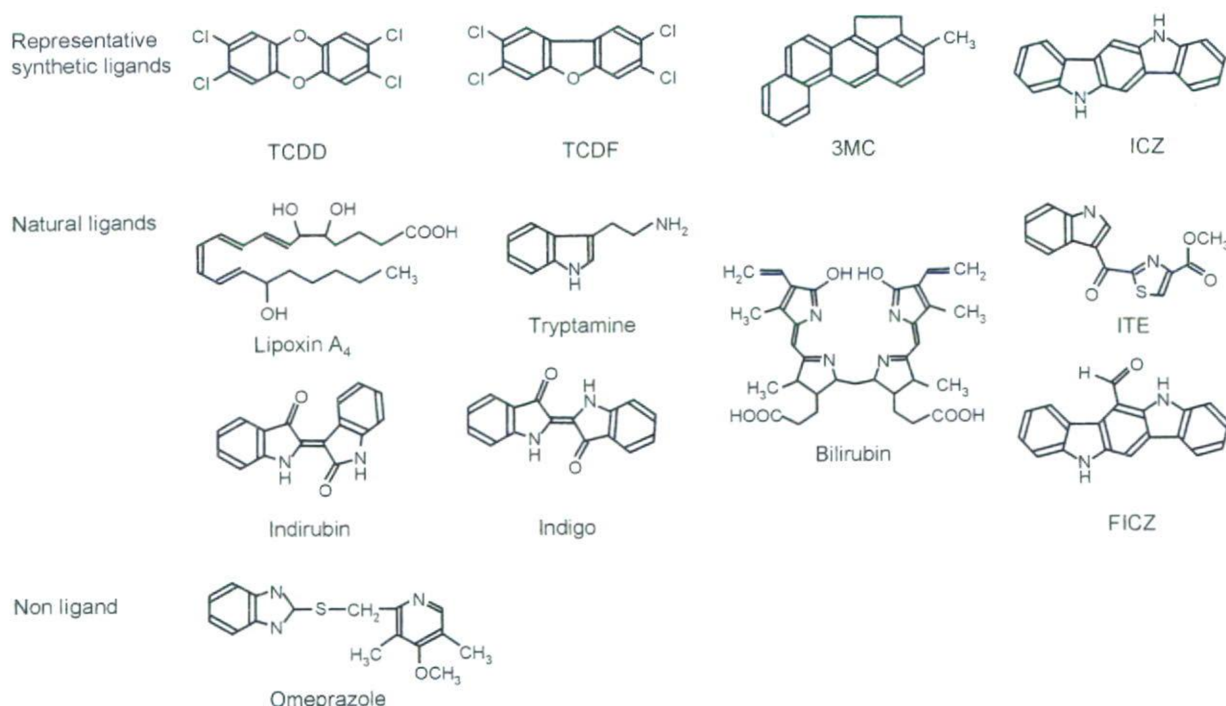


Fig. 1. Chemical activators of AhR. TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDF: 2,3,7,8-tetrachlorodibenzofuran, 3MC: 3-methylcholanthrene, ICZ: indro[3,2-*b*]carbazole, ITE: 2-(1′*H*-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester, FICZ: 6-formylindolo[3,2-*b*]carbazole.

reporter at levels comparable to TCDD stimulation of confluent cells in a manner independent of cell cycle changes. Suspension culture and TCDD treatment induced comparable AhR nuclear translocation and AhR/Arnt complex formation [22]. Culture of a keratinocyte cell line, HaCaT, at low cell densities or at confluence in Ca^{2+}-deficient S-MEM induces the nuclear accumulation of AhR in association with enhanced expression of a reporter gene whose expression is driven by XRE sequences [23]. These findings suggest that disruption of cell–cell contacts or cell–cell interactions stimulates the nuclear localization and transcriptional activity of AhR via a signal transduction pathway. The nuclear accumulation of AhR is regulated by the phosphorylation of Ser68 within the nuclear export sequence (NES) of AhR. Use of specific kinase inhibitors has suggested that this phosphorylation event is catalyzed by p38 MAPK [23]. An increasing number of reports have described that phosphorylation regulates AhR activity in the physiological signaling pathway regulating cell cycle progression as well as the xenobiotic signal transduction pathway [24]. Omeprazole induces CYP1A1 in an AhR-dependent manner without binding directly to AhR [25,26]. Tyrosine kinase inhibitors, tyrphostins AG17 and AG879, selectively inhibited omeprazole-mediated AhR signaling, but did not affect TCDD-mediated induction of CYP1A1. Mutational analysis provided evidence that a Tyr320Phe mutation abolished omeprazole-dependent AhR activation, while the TCDD-dependent activation of *CYP1A1* transcription was only minimally affected. These results suggest that Tyr320 is a putative phosphorylation site on AhR activated by omeprazole in a ligand-independent manner via a signal transduction pathway that involves protein tyrosine kinases. This pathway is independent from that induced by high-affinity ligands, such as TCDD [26]. Although the protein kinases involved remain unclear, AhR can be activated in a ligand-independent manner.

Cis-acting DNA elements

The regulatory DNA elements responsible for the induction of CYP1 by polyaromatic hydrocarbons like TCDD, called XREs, were first identified by transient DNA transfection experiments using a reporter gene, whose expression was driven by the *CYP1A1* promoter [27]. Later, additional experiments introducing a variety of mutations at this locus defined the consensus sequence and designated this sequence the DRE or AhRE [5,28]. All *CYP* genes whose expression are induced by PAH or HAH, including CYP1A1, 1A2 [6,27,28], 1B1 [29], 2A8 [30], and 19 [31] (Fig. 2), carry XRE sequences within their promoters. The human *CYP1A1* and *1A2* genes, found on chromosome 15q23 [32], are arranged in head to head orientation at a distance of approximate 23 kb apart. A similar chromosomal arrangement is reported for the mouse *Cyp1A1* and *1A2* genes on chromosome 9B, the syntenic chromosome to human Ch15q23. Although both genes are similarly inducible by PAH or HAH and share a common 5′ flanking region, the regulatory mechanisms governing the expression of each are quite different. Under normal conditions, CYP1A2 is expressed at basal levels in the liver, while no basal expression of CYP1A1 is observed in this

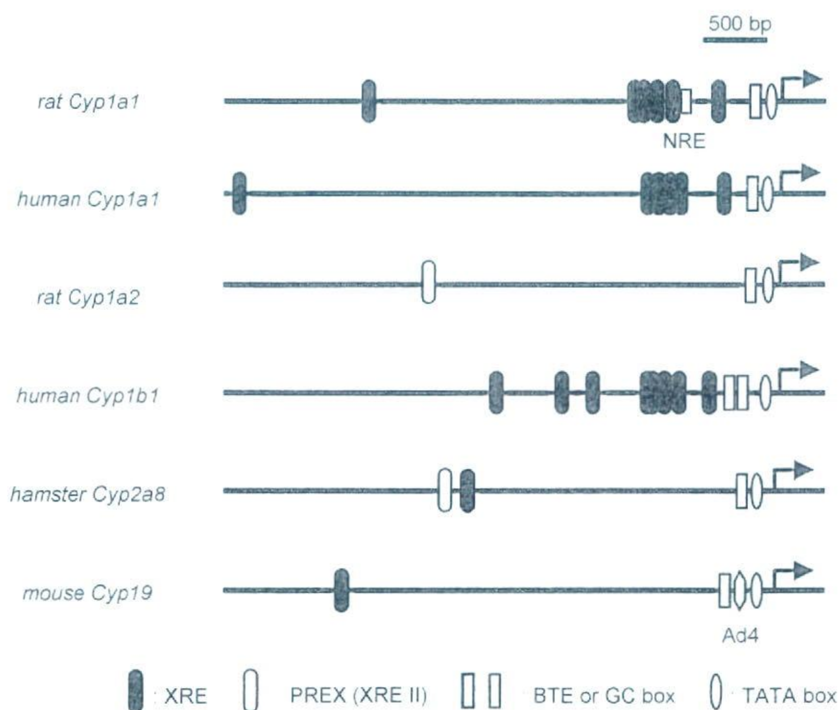


Fig. 2. Schematic representation of regulatory elements in the promoter of *CYPs*.

organ. Expression of CYP1A1 could be upregulated in multiple tissues in response to inducers, while that of CYP1A2 is restricted to the liver. In AhR-deficient mice, CYP1A1 expression is completely abolished; in these animals, although basal expression of CYP1A2 was retained in the liver, inducible expression was lost [33]. These findings clearly indicate that the expression of CYP1A1 and 1A2 is differently regulated, despite a common 5′ upstream sequence. Recently, a DNA element responsive to 3MC (XRE2: CATGN_6 CTTG), which is similar to a consensus DNA-binding sequence [CNRG-$N_{5\sim6}$-CNR(G/C)] recognized by the LBP-1 family, was discovered in the proximal promoter of the *CYP1A2* gene [34]. A putative factor (X) binding to this sequence was suggested by GMSA. Ligand-activated AhR/Arnt bound to the X factor as a coactivator is likely conferring inducibility of the *CYP1A2* gene in response to the inducer [34]. Coactivator-like functions of AhR/Arnt have already been reported [35,36]. An analogous sequence, designated PREX, was also identified within the promoter of the *CYP2A8* gene. This sequence acted as an inducible enhancer that cooperated with the XRE sequence in the *CYP2A8* promoter. The factor binding to PREX was identified as NF2d9 (LBP-1a) [37]. Less is known of the regulatory mechanisms governing the inducible expression of CYP1A2. In addition to the XRE sequence, a BTE (basic transcription element) sequence, which is a GC box sequence localized in the proximal promoter of *CYP1A1*, is also important to achieve a high level of CYP1A1 [38] and 2A8 [30] inducible expression. The XRE and GC box sequences frequently coexist in the promoters of xenobiotic-inducible genes, suggesting that these elements cooperatively enhance the inducible expression of these genes (Figs. 2 and 3). Another putative NF-1 binding site has also been reported within the proximal promoter of the *CYP1A1* gene [9], but no experimental evidence has addressed its functional significance. Two regulatory DNA elements are found in the promoter of *CYP19* gene, XRE and Ad4/SF-1, whose cognate binding factors, AhR and Ad4BP/SF-1, interact on the chromosomal DNA to enhance gene expression synergistically [31].

Trans-acting factor for XRE

Mouse genetics initially implicated the existence of a mediator of the xenobiotic signaling, leading to the induction of CYP1A1 expression. This mediator was later identified as a factor binding to xenobiotics, which was designated the aryl hydrocarbon receptor or AhR [39]. This factor was also dubbed the dioxin receptor (DR), due to the high avidity with which it bound TCDD. Approximately a decade later, GMSA revealed that a factor that bound the XRE sequence in a TCDD-dependent manner contained a factor which also bound TCDD directly. This XRE-binding factor behaved like AhR; both the cytoplasmic localization under normal conditions and the induction of nuclear translocation by TCDD treatment mimicked the patterns seen for AhR [40]. cDNA cloning of this XRE-binding factor revealed its molecular structure to be a DNA-binding tran-

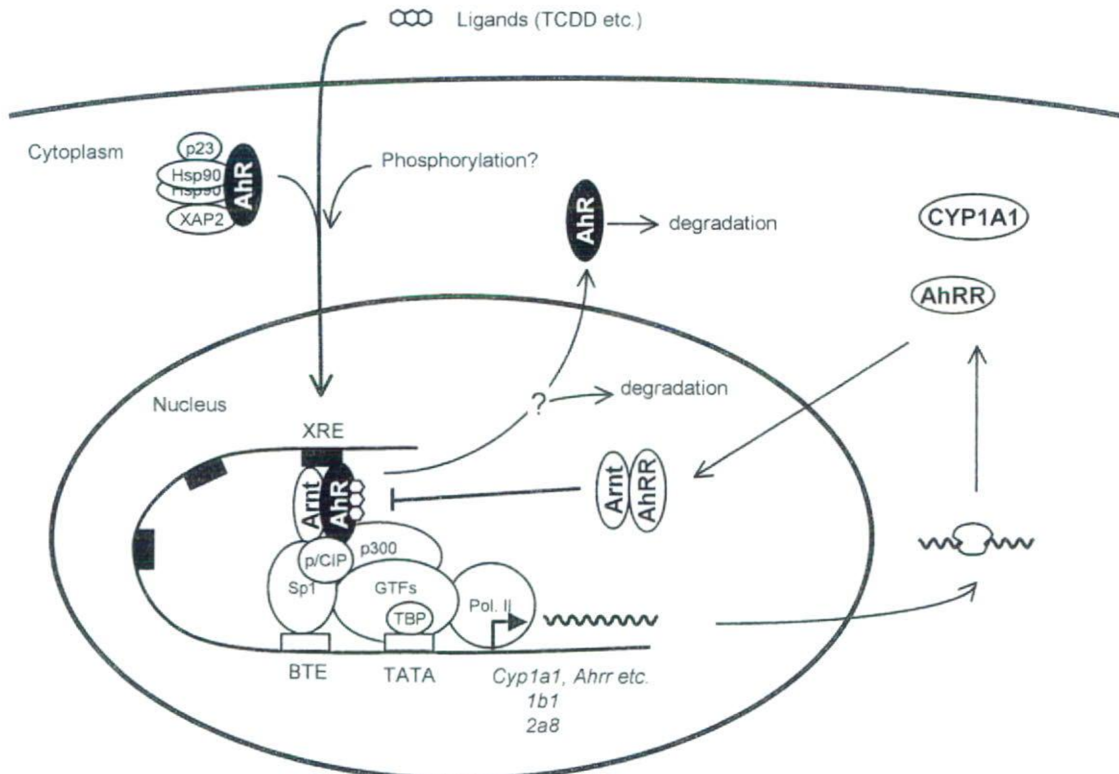


Fig. 3. A model of AhR signaling pathway.