treated with biotinylated anti-mouse IgG antibody (Vector) for 3 h at 4 °C, washed with PBS, and treated with streptavidin-Alexa Fluor 488 (Invitrogen) and Hoechst solution (Dojindo) for 30 min at room temperature. After washing with PBS, a drop of fluorescent mounting medium (Dako) was placed on the cells, which were then examined by fluorescence microscopy.

Real-time PCR—Total RNA samples were prepared from the treated cells using Isogen (Nippon Gene) as described. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was performed in triplicate for each sample with the ABI Prism 7700 sequence detector (PE Applied Biosystems) using primers designed against mouse *GLUT1* (26) and *CYP1A1* (primer sequences GGTACAGAGAAAGATCCAGGAGGA and CGAAGGATGAATGCCGGAAGGTCT and probe sequence 6-FAM-CTAGACACAGTGATTGGCAGAGATCGGCA-TAMRA) or rRNA genes (PE Applied Biosystems).

RESULTS

Comparison between Transcription Activities of Arnt and Arnt2 for Expression of the XRE- and HRE-driven Reporter Genes—It has been reported that the bHLH and PAS domains of Arnt and Arnt2 are very similar and mediate homo- and heterodimerization (Fig. 1*A*), but Arnt2 showed only 20% transcription activity of Arnt in the expression of the XRE-driven reporter gene (13).

To clarify whether the low transcription activity of Arnt2 for the expression of the XRE reporter gene is because of a low level of expression of Arnt2 or a low affinity of Arnt2 to AhR as compared with Arnt, we investigated the transcription activity of the XRE- and HRE-driven reporter genes by Arnt2 and Arnt in a dose-dependent manner using the transient transfection assay. In an experiment using either Hepa1-c4 or HeLa cells (the same result was obtained with NIH3T3 cells, data not shown), a highly inducible expression of the XRE-driven reporter gene was observed by Arnt in response to 3MC. In marked contrast, Arnt2 exhibited only a low level of inducible expression of the reporter gene even at the highest dose (Fig. 1, *B* and *D*). On the other hand, the HRE-driven reporter gene was induced similarly by Arnt and Arnt2 in response to hypoxia. Taken together with the results that Arnt and Arnt2 were similarly expressed at a protein level as shown in Fig. 1, *C* and *E*, all of these results suggest that Arnt2 is much less efficient than Arnt in conjunction with AhR for the inducible expression of the XRE reporter gene, whereas both Arnt and Arnt2 work equally well with HIFα to regulate the HRE reporter gene expression.

PAS Domain Is Responsible for Differential Activities between Arnt and Arnt2 for XRE-driven Reporter Gene Expression—We were interested in the molecular mechanisms responsible for the differences in transcriptional activity between Arnt and Arnt2. To investigate which part of the Arnt and Arnt2 molecules is responsible for the differential activity, we divided Arnt and Arnt2 into three parts based on the N-terminal bHLH, PAS, and C-terminal activation domains and generated several chimeric constructs by swapping the respective domains for Arnt and Arnt2 (Fig. 2*A*). As shown in the *upper panel* of Fig. 2*B* (*column 1 versus 3*), the luciferase activity is the same in cells

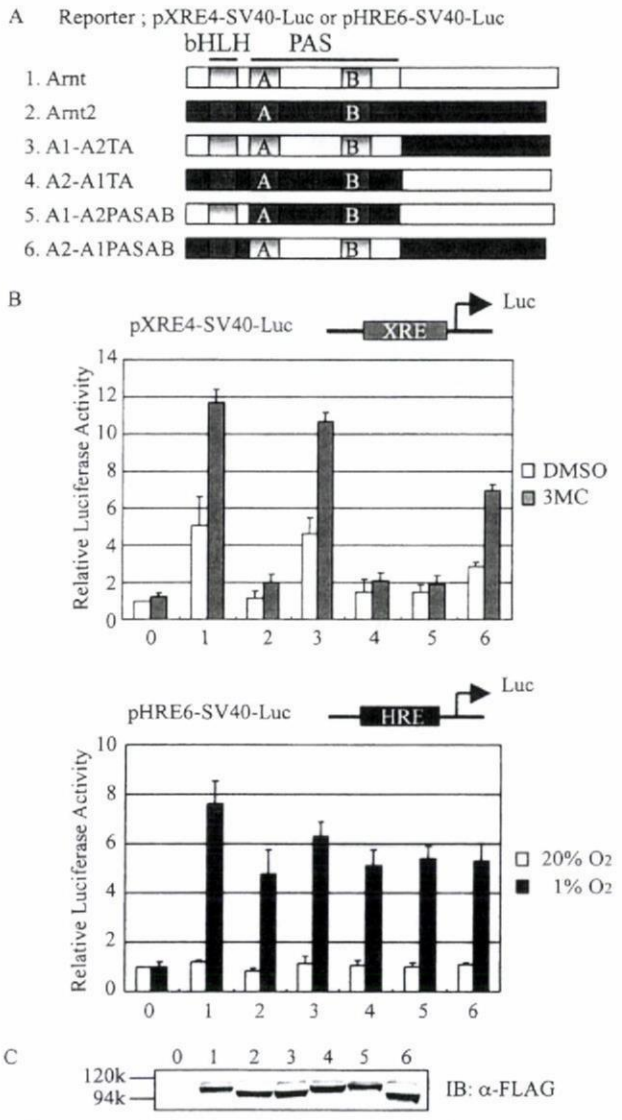
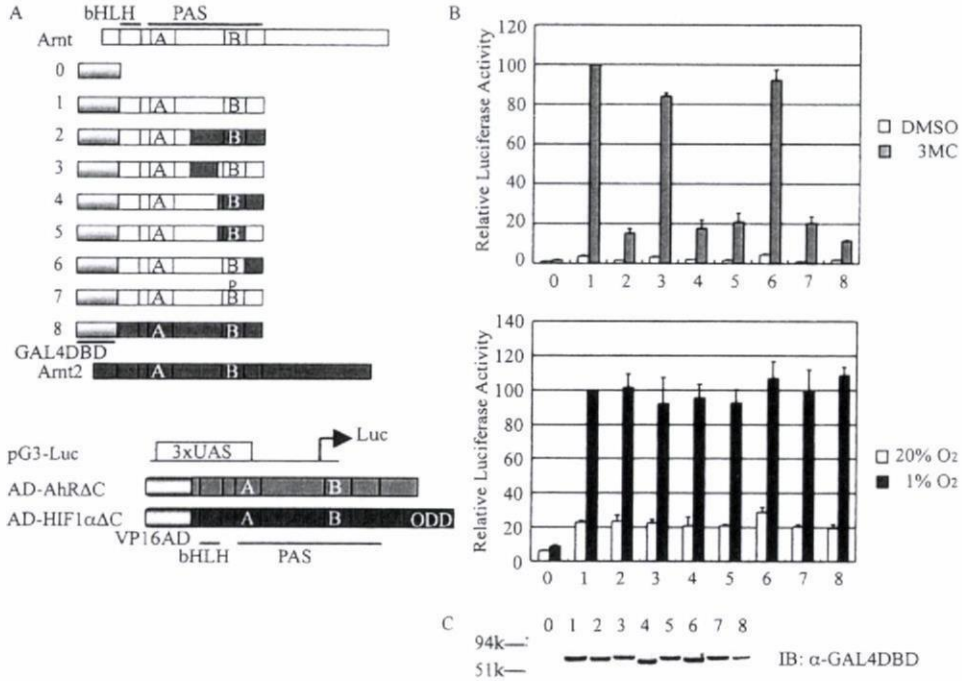


FIGURE 2. **Transcriptional activity of Arnt/Arnt2 chimeric proteins on XRE- and HRE-driven reporter genes in Hepa1-c4 cells.** *A*, Arnt and Arnt2 structures and their domain-swapping constructs. *B*, transcriptional activity of Arnt/Arnt2 chimeric constructs. Hepa1-c4 cells transfected with 100 ng of pXRE4-SV40-Luc and 10 ng of the indicated Arnt/Arnt2 chimeric constructs were incubated for 24 h and then treated with 1 μM 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) for 18 h (*top panel*). For analysis of the hypoxic response, Hepa1-c4 cells were transfected with 100 ng of pHRE6-SV40-Luc and 10 ng of the Arnt/Arnt2 chimeric constructs. After 2 h of incubation, the cells were treated for 16 h under normoxia or hypoxia (1% O_2) (*bottom panel*). The cell extracts were prepared from the treated cells and used for luciferase assays. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. *C*, expression of Arnt, Arnt2, and chimeric constructs. The cells were transfected with 50 ng of the indicated expression construct in 6-well plates. The protein levels of all constructs were evaluated by Western blotting using anti-FLAG antibody. Equal amounts of cell lysates were used for Western blot analysis. *Columns* in *B* and *C*: *0*, pBOS; *1*, pBOS3xFLAG-Arnt; *2*, pBOS3xFLAG-Arnt2; *3*, pBOS3xFLAG-A1(A2TA); *4*, pBOS3xFLAG-A2(A1TA); *5*, pBOS3xFLAG-A1(A2AB); *6*, pBOS3xFLAG-A2(A1AB). *IB*, immunoblot.

expressing WT Arnt and a chimera composed of the N-terminal bHLH and PAS domains of Arnt and the C-terminal region of Arnt2. In contrast, a chimera composed of the bHLH and C-terminal domains of Arnt and the PAS domain of Arnt2 activates only a low level of luciferase expression, as observed with WT Arnt2 for the expression of XRE-driven reporter gene (Fig.



D

		βE	βA
mArnt	334	KFCLVAIGRLQVTSSPNCTDMSNICQPTEFISRHNIEG	
hArnt	334	KFCLVAIGRLQVTSSPNCTDMSNVCQPTEFISRHNIEG	
cArnt	355	KFCLVAIGRLQVTSSPNCTDMSNVCQPTEFISRHNTEG	
xArnt	262	KFCLVAIGRLQVTSSPTCTDINTVCQPIEFISRHSVNG	
zArnt	309	RFCLVAIGRLQVTCCPSDTSINNISVPVEFISRHNSQG	
mArnt2	308	KYCLVAIGRLQVTSSPVCMDMSGMSVPTEFLSRHNSDG	
hArnt2	308	KYCLVAIGRLQVTSSPVCMDMNGMSVPTEFLSRHNSDG	
xArnt2	307	KYCLVAIGRLQVTSSPGCLDMNGMSLPTEFLSRHNTEG	
zArnt2	323	KYCLVAIGRLQVTSSPVSMDMNGLSVPTEFLSRHNSDG	

		βB	αA	αB
mArnt	372	IFTFVD	H	RCVATVGYQPQELLGKNIV
hArnt	372	IFTFVD	H	RCVATVGYQPQELLGKNIV
cArnt	383	IFTFID	H	RCVATVGYQPQELLGKDIV
xArnt	300	LFTFVD	H	RCTATVGYQPQELLGKDIV
zArnt	347	VYTFVD	H	RCTATVGFQTQELLGKNIL
mArnt2	346	IITFVD	P	RCISVIGYQPQDLLGKDIL
hArnt2	346	IITFVD	P	RCISVIGYQPQDLLGKDIL
xArnt2	345	VITFVD	P	RCISIIGYQPQDLLGKDII
zArnt2	361	IITFVD	P	RCTNVIGYQPQDLLGKDIL

FIGURE 3. **Two-hybrid analysis of transcriptional activities of Arnt and Arnt2 and their chimeric and mutant derivatives.** *A*, constructs of bait proteins of Arnt and Arnt2, their chimeras and mutant, and prey molecules of AhR and HIF1α. *B*, transcriptional activity of various Arnt/Arnt2-bHLHPAS chimeric proteins in the two-hybrid system. 293T cells were transfected with pG3-Luc, the indicated Arnt/Arnt2 construct, and pBOSVP16AD-mAhRΔC (*AD-AhRΔC*) or pBOSVP16AD-mHIF1αΔC (*AD-HIF1αΔC*) (see "Experimental Procedures"). The transfected cells were treated as described in the legend to Fig. 1*B*. *Top panel*, relative luciferase activities induced by 3MC treatment. *Bottom panel*, relative luciferase activities induced by hypoxic treatment. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as internal control. *DMSO*, dimethyl sulfoxide (Me_2SO). *C*, expression of the bait proteins. The cells were transfected with 50 ng of the indicated expression construct in 6-well plates. Protein levels of all constructs were evaluated by Western blot analysis using α-GAL4DBD antibody. The cells were homogenized, and supernatants were subjected to SDS-PAGE for Western blot analysis (*IB*, immunoblot). *Columns*: *0*, pBOSGAL4DBD; *1*, pBOSGAL4DBD-Arnt-bHLHPAS; *2*, pBOSGAL4DBD-Arnt2-bHLHPAS; *3*, pBOSGAL4DBD-A1A2-bHLHPAS; *4*, pBOSGAL4DBD-A1A2A1–1-bHLHPAS; *5*, pBOSGAL4DBD-A1A1A2–1-bHLHPAS; *6*, pBOSGAL4DBD-A1A2A1–2-bHLHPAS; *7*, pBOSGAL4DBD-A1A1A2–2-bHLHPAS; *8*, pBOSGAL4DBD-ArntH378P-bHLHPAS (see "Experimental Procedures"). The full activity of *column 1* was taken as a standard to calculate relative activities. *D*, amino acid sequences of a part of PASB region of Arnt and Arnt2 responsible for differential transcriptional activities in association with AhR. Predicted exposed side chains of amino acids (27) are indicated by *arrows*. *m*, mouse; *h*, human; *c*, chicken; *x*, *Xenopus*; *z*, zebrafish.

2*B*, *upper panel*, *columns 2* and *5*). Thus, it is not the highly variable C-terminal domain but the PAS domain that is responsible for the differences in XRE-driven luciferase expression. In contrast, any combination of Arnt and Arnt2 domains was able to activate luciferase expression to the same extent in response to hypoxia. Taken together, these results suggest that only the PAS domain of Arnt is capable of efficient heterodimerization with AhR to activate the XRE, but HIFα interacted equally with the PAS domains of Arnt and Arnt2 leading to HRE-mediated transcription.

Interaction of the PAS Domains of Arnt or Arnt2 with Those of AhR and HIF1α—To further investigate the interaction of the PAS domains of Arnt and Arnt2 with AhR, we used a mammalian two-hybrid system. Arnt/Arnt2 bHLH-PAS chimeric constructs fused with the GAL4DBD were used as bait, and AhRΔC or HIF1αΔC fused with the VP16 activation domain were used as preys (Fig. 3*A*). Replacement of only a small portion of the PASB domain of Arnt with that of Arnt2 almost completely abrogated 3MC-induced luciferase expression (Fig. 3*B*, *upper panel*, *columns 2*, *4*, and *5 versus 1*, *3*, and *6*). All constructs were equally expressed except for a slightly lower expression of construction 8 (Fig. 3*C*). Taken together, these data indicate that the critical region for determining the differential interaction of Arnt and Arnt2 with AhR covers the sequence of amino acids 334–397 of Arnt and 308–371 of Arnt2. This region is predicted to form the N-terminal cap and a part of the PAS core of the PASB fold and is thought to be solvent-exposed, forming an interface to interact with partner proteins (27). In this region there are seven amino acids with side chains that are predicted to be solvent-exposed (27) (indicated by *arrows* in Fig. 3*D*), and six are conserved or conservative amino acid replacements between Arnt and Arnt2. Only the replacement of His with Pro was considered to be significant for the functional difference between Arnt and Arnt2. These amino acids, His and Pro, are conserved, respectively, in Arnt and Arnt2 of various animal species (Fig. 3*D*). When we generated a His-to-Pro point mutation in Arnt, this construct was not able to interact efficiently with AhR to activate luciferase expression in response to 3MC (Fig. 3*B*, *upper panel*, *column 7*). On the other hand, all chimeric constructs, WT Arnt and Arnt2, and

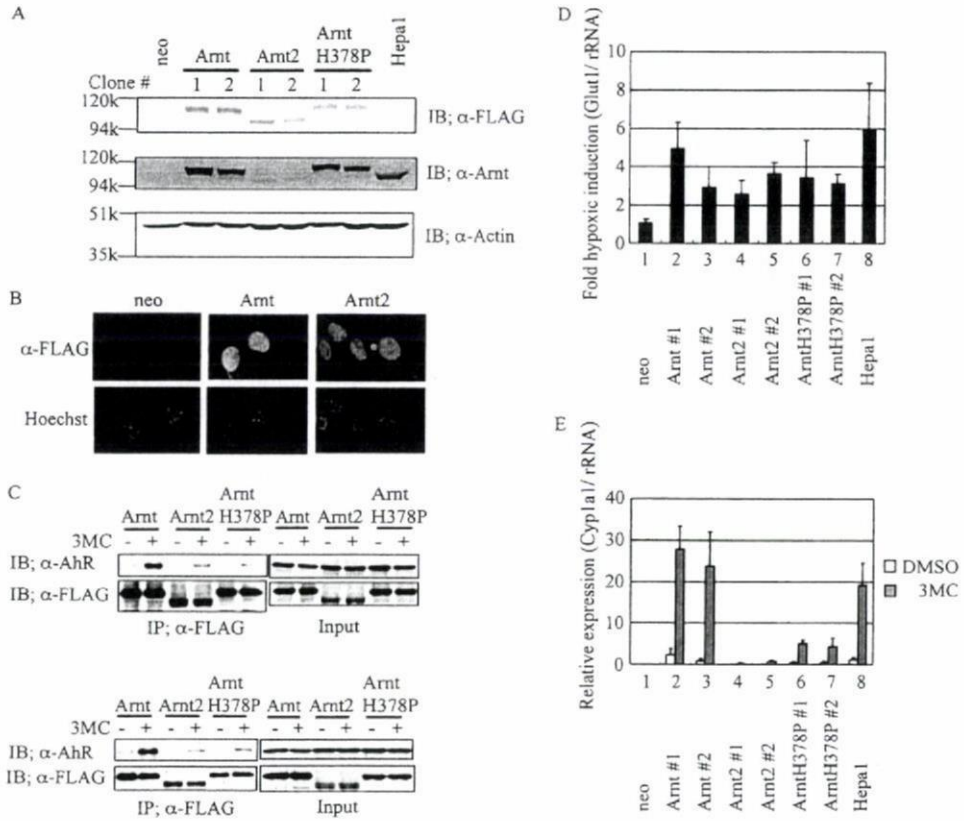


FIGURE 4. **Transcriptional activities of Arnt, Arnt2 and ArntH378P mutant in stably transformed Hepa1-c4 cells.** *A*, expression of 3xFLAG-Arnt, -Arnt2, and -ArntH378P. Expressed proteins were assessed by Western blotting (*IB*, immunoblot) using anti-Arnt, anti-FLAG, and anti-actin antibodies. *B*, cellular localization of 3xFLAG-Arnt and 3xFLAG-Arnt2. Hepa1-c4 cells stably expressing 3xFLAG-Arnt and 3xFLAG-Arnt2 were fixed and immunostained for Arnt and Arnt2 with anti-FLAG antibody. *C*, coimmunoprecipitation of AhR with Arnt, Arnt2, and ArntH378P in response to 3MC. Whole cell extracts of transformants expressing 3xFLAG-Arnt, 3xFLAG-Arnt2, and 3xFLAG-ArntH378P treated with 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) were coimmunoprecipitated by anti-FLAG antibody. Coimmunoprecipitation and Western blotting were performed by the methods described under "Experimental Procedures." The *top* and *bottom left panels* show AhR and 3xFLAG-Arnts proteins coimmunoprecipitated by anti-FLAG antibody. *Input* is shown in the *right panels*. The *upper* and *lower panels* represent clone 1 and clone 2, respectively. *D*, expression of endogenous *Glut-1* gene in transformants expressing 3xFLAG-Arnt, -Arnt2, and -ArntH378. Transformed Hepa1-c4 cells were cultured for 16 h under conditions of normoxia or hypoxia (1% O_2), and cell extracts were prepared and used for determination of Glut-1 mRNA expression by real-time PCR analysis. Values are normalized against those for rRNA, and the results are expressed as induction ratios of hypoxic to normoxic activities. *E*, Expression of endogenous *CYP1A1* gene in the transformants. Stably transformed Hepa1-c4 cells were treated with Me_2SO or 3MC for 18 h, and the cell extracts were prepared and used for determination of CYP1A1 mRNA by real-time PCR analysis.

the Arnt His-to-Pro mutant induced luciferase expression to the same extent in response to hypoxia (Fig. 3*B*, *lower panel*). These data indicate that a single amino acid change is mainly responsible for the differential binding of Arnt and Arnt2 with AhR, whereas both Arnt and Arnt2 are equally capable of binding with HIFα.

Physical Interaction and Transcriptional Activity of Arnt and Arnt2 with AhR in Stable Transformants—Up to this point, we have studied the activity and interactions of WT Arnt and Arnt2 and their mutants with AhR and HIFα using relatively artificial transient transfection and two-hybrid assays. We wanted to examine the behavior of these proteins in a more physiologic setting, and we generated Hepa1-c4 cells stably expressing 3xFLAG-Arnt, -Arnt2, and -ArntH378P as described under "Experimental Procedures."

For each construct we isolated two clones with 3xFLAG-Arnt/Arnt2 expression levels comparable with endogenous Arnt in unmodified Hepa1 cells (Fig. 4*A*) and the stably expressed 3xFLAG-Arnt, -Arnt2, and -ArntH378P (data not shown) proteins localized to the nucleus (Fig. 4*B*) (28). To evaluate the interaction between endogenous AhR and these stably expressed 3xFLAG-Arnt, -Arnt2, and -ArntH378P proteins, AhR-3xFLAG-Arnt coimmunoprecipitation assays were performed. In the absence of cellular treatment with 3MC, AhR was not coimmunoprecipitated with 3xFLAG-tagged Arnt, Arnt2, or ArntH378P by an anti-FLAG antibody. However, following incubation of cells with 3MC, AhR was coimmunoprecipitated with 3xFLAG-Arnt by using anti-FLAG antibody, but only a small amount of AhR was detected in the anti-FLAG immunoprecipitates from two transformant cell lines expressing 3xFLAG-Arnt2 or 3xFLAG-ArntH378P (Fig. 4*C*). These results are consistent with the reduced affinity of Arnt2 or the ArntH378P mutant for AhR as revealed in the mammalian two-hybrid system (Fig. 3*B*, *top panel*, *columns 1*, *7*, and *8*), but they are at odds with a previous study from our laboratory (13). In that report, we incubated *in vitro* translated Arnt and Arnt2 with AhR produced in Sf2 cells. It is likely that the relatively high concentrations of Arnt2 and AhR produced in the *in vitro* incubation system gave rise to a misleadingly significant band.

We next investigated the transcriptional activity of Arnt, Arnt2, and the ArntH378P mutant in the generated stable transformant cells, and we chose to examine Glut-1 and CYP1A1 induction by quantitative real-time PCR as target genes for HIF1α and AhR, respectively (29, 30). In cells expressing 3xFLAG-Arnt, -Arnt2, or -ArntH378P, Glut-1 was induced to a similar extent under hypoxic conditions when mRNA levels were normalized to normoxic cells (Fig. 4*D*). In contrast, treatment of cells stably expressing 3xFLAG-Arnt with 3MC dramatically increased CYP1A1 mRNA levels compared with untreated cells, and little induction was seen in cells expressing 3xFLAG-Arnt2 (Fig. 4*E*). Cells expressing 3xFLAG-ArntH378P slightly, but significantly, increased CYP1A1 mRNA levels following 3MC treatment, indicating that His-378 in the Arnt PASB domain strongly influences Arnt binding to AhR but that other regions of Arnt also contribute to AhR binding.

The Arnt PASA and PASB Domains Cooperatively Bind AhR—We saw some degree of *CYP1A1* transcription in response to 3MC in cells expressing 3xFLAG-ArntH378P, and we were

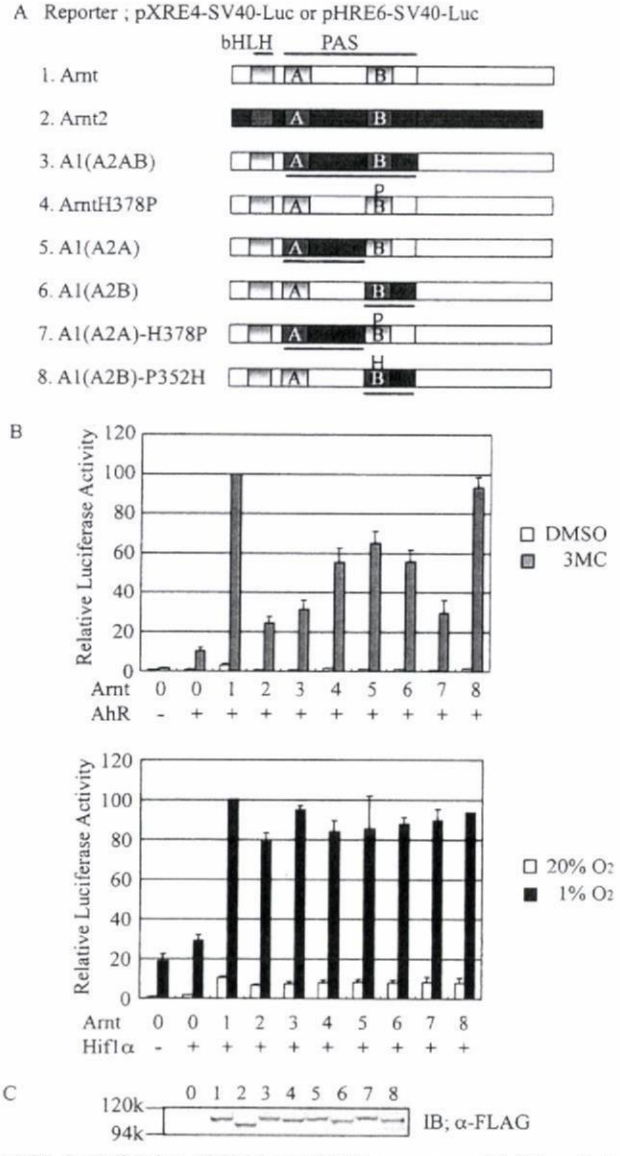


FIGURE 5. **Contribution of PASA and PASB sequences of full-length Arnt and Arnt2 to the expression of XRE-driven and HRE-driven reporter genes.** *A*, constructs of the expression plasmids of Arnt and Arnt2 and their chimeric and mutant proteins. *Underlines* show the Arnt domain replaced by the corresponding Arnt2 domain (*constructs 3* and *5–8*). *B, top*, expression of the XRE-driven reporter gene. HeLa cells were transfected with 10 ng of pXRE4-SV40-Luc, 2 ng of the indicated expression plasmid, and 20 ng of pBOSmAhR, and the reporter gene expression assay was performed as described in the legend to Fig. 1*B*. *DMSO*, dimethyl sulfoxide (Me_2SO). *Bottom*, expression of the HRE-driven reporter gene. HeLa cells were transfected with 10 ng of pHRE6-SV40-Luc, 2 ng of the indicated expression plasmid, and 20 ng of pBOShHIF1α, and the reporter gene expression assay was performed as described in the legend to Fig. 1*B*. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. The full activity of *column 1* was taken as a standard to calculate relative activities. *C*, concentrations of the expressed effector proteins. The cells were transfected with 10 ng of the indicated expression construct in 6-well plates. Equal amounts of cell lysates were used for determination of the expressed proteins. *Columns* in *B* and *C*: *0*, pBOS; *1*, pBOS3xFLAG-Arnt; *2*, pBOS3xFLAG-Arnt2; *3*, pBOS3xFLAG-A1(A2AB); *4*, pBOS3xFLAG-ArntH378P; *5*, pBOS3x-FLAG-A1(A2A); *6*, pBOS3xFLAG-A1(A2B); *7*, pBOS3xFLAG-A1(A2A)H378P; *8*, pBOS3xFLAG-A1(A2B)P352H. *IB*, immunoblot.

interested in determining other regions of Arnt that support its interaction with AhR. Toward this end, we generated several additional Arnt/Arnt2 chimeric constructs (Fig. 5*A*) and investigated their ability to interact with AhR by luciferase reporter assay. As shown above, all WT and chimeric Arnt and Arnt2 constructs were able to induce luciferase expression in response to hypoxia when coexpressed with HIF1α (Fig. 5*B*, *lower panel*). As expected, Arnt induced XRE-driven luciferase expression to a greater extent than Arnt2, but some luciferase expression was seen in Arnt2-expressing cells compared with untreated cells as reported in our previous study (13) (Fig. 5*B*, *upper panel*, *columns 0*, *1*, and *2*). The physiologic significance of this induction is questionable, however, given the inability of stably expressed Arnt2 to induce the expression of the endogenous *CYP1A1* gene (Fig. 4*E*, *columns 4* and *5*); the ability of Arnt2 to induce luciferase in response to 3MC may be an overexpression artifact. Substitution of the Arnt PASA and PASB domains with those of Arnt2 led to reduced luciferase expression comparable with WT Arnt2 (Fig. 5*B*, *upper panel*, *columns 2*, and *3*). Additionally, the H378P mutation as well as swapping the PASA or PASB domain of Arnt2 led to intermediate levels of luciferase expression (Fig. 5*B*, *upper panel*, *columns 4*, *5*, and *6*), indicating that the PASA domain is largely responsible for the additional AhR binding activity of Arnt. Interestingly, replacement of the Arnt PASA domain with that of Arnt2 and mutation of His-378 to Pro led to luciferase expression comparable with that of WT Arnt2 (column 7), thus confirming the importance of the two regions for Arnt·AhR binding. In contrast, when the Pro residue of the Arnt2 PASB domain was mutated to His, and this mutant PASB domain replaced the corresponding one in Arnt, luciferase expression in response to 3MC was restored to WT Arnt levels (Fig. 5*B*, *upper panel*, *column 8*). As expected from the two-hybrid assay, all WT and chimeric Arnt/Arnt2 constructs behaved similarly in response to hypoxia (Fig. 5*B*, *lower panel*).

The PASB Domain of AhR Is Responsible for Differential Binding to Arnt and Arnt2—A single amino acid change largely impaired the ability of Arnt to bind AhR, and we determined the region of AhR that interacts with Arnt PASB. We used a mammalian two-hybrid system with GAL4DBD-fused Arnt, Arnt2, and the Arnt mutant as bait (Fig. 3*A*, *constructs 1*, *7*, and *8*). When we used VP16AD-AhRΔC (Fig. 6*A*) as prey together with GAL4DBD-Arnt-bHLHPAS, the reporter gene was strongly induced following 3MC treatment (Fig. 6*B*, *column 4*). Expression of a VP16AD-AhRΔBΔC construct (Fig. 6*A*) further lacking the AhR PASB region constitutively expressed a high degree of luciferase activity, although a slight inducibility remained with 3MC (Fig. 6*B*, *column 5*). The PASB region of AhR binds the HSP90 complex, and in its absence, AhR is not retained in the cytoplasm and mediates transcription in the absence of stimuli (31–33). In contrast, in cells expressing both GAL4DBD-Arnt2-bHLHPAS and VP16AD-AhRΔC, reporter gene expression was reduced remarkably and was only slightly inducible (Fig. 6*B*, *column 10*). In stark contrast, coexpression of the PASB-deleted construct VP16AD-AhRΔBΔC with GAL4DBD-Arnt2-bHLHPAS led to constitutive luciferase activity comparable with that seen with GAL4DBD-Arnt-bHLHPAS (Fig. 6*B*, *column 5 versus 11*). These results indicate that the bHLH and PASA domains of AhR are able to interact with Arnt and Arnt2 to an equal extent to mediate constitutive transcriptional activity. Addition of the PASB region of AhR, how-

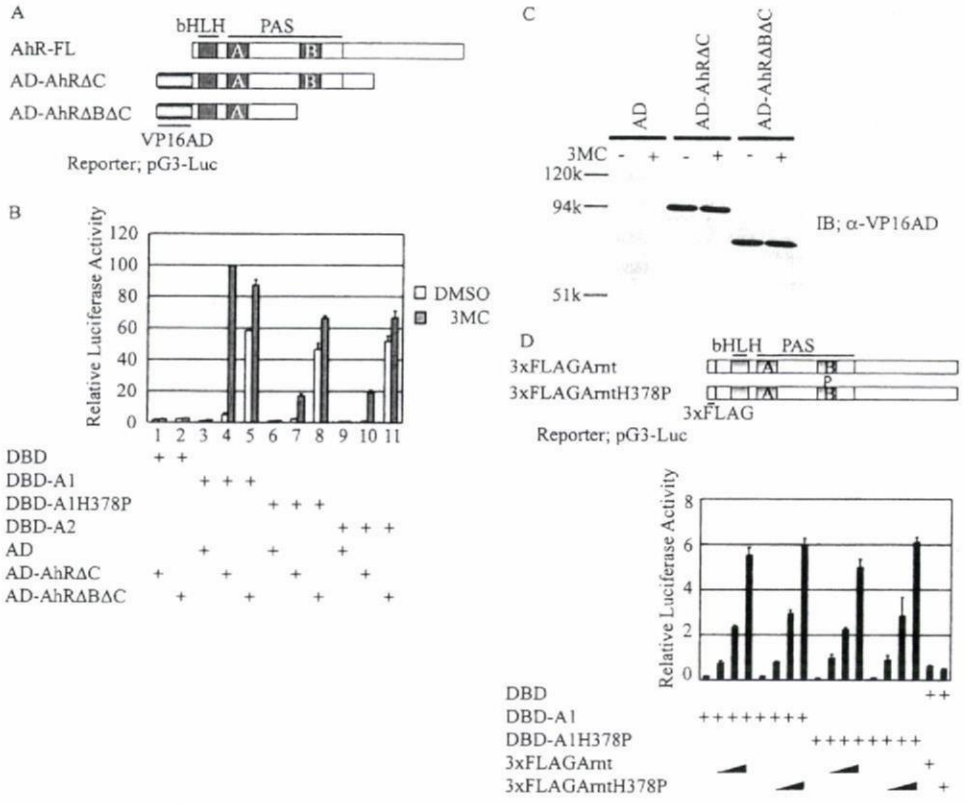


FIGURE 6. **PASB/PASB interaction is important for specific AhR/Arnt heterodimerization.** *A*, constructs of VP16AD-AhRΔC (*AD-AhRΔC*) and VP16AD-AhRΔBΔC (*AD-AhRΔBΔC*) used as prey. *B*, reporter gene expression. 293T cells were transfected with pG3-Luc, the indicated bait plasmid (pBOSGAL4DBD (*DBD*), pBOSGAL4DBD-Arnt-bHLHPAS (*DBD-A1*), pBOSGAL4DBD-ArntH378P-bHLHPAS (*DBD-A1H378P*), or pBOSGAL4DBD-Arnt2-bHLHPAS (*DBD-A2*)), and the indicated prey plasmid (pBOSVP16AD (*AD*), pBOSVP16AD-mAhRΔC, or pBOSVP16AD-mAhRΔBΔC), and after 24 h of incubation, the cells were treated with 1 μM 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) for 18 h. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. The full activity of *column 1* was taken as a standard to calculate relative activities. *C*, protein expression. The cells were transfected with 50 ng of the indicated expression construct in 6-well plates. The protein levels of all constructs were evaluated by Western blotting (*IB*, immunoblot) using anti-VP16AD antibody. Equal amounts of cell lysates were used for gel electrophoresis. *Columns 1* and *2*, pBOSVP16AD (*AD*); *columns 3* and *4*, pBOSVP16AD-mAhRΔC (*AD-AhRΔC*); *columns 5* and *6*, pBOSVP16AD-mAhRΔBΔC (*AD-AhRΔBΔC*). In *columns 1*, *3*, and *5*, cells were treated with Me_2SO for 18 h; in *columns 2*, *4*, and *6*, cells were treated with 1 μM 3MC for 18 h. *D*, effect of H378P mutation on Arnt/Arnt homodimerization. *Top*, 3xFLAG-Arnt and 3xFLAG-ArntH378P were used as activators. *Bottom*, expressed luciferase activities from Arnt/Arnt interaction. 293T cells were transfected with 100 ng of pG3-Luc and 10 ng of the indicated bait vectors (pBOSGAL4DBD, pBOSGAL4DBD-Arnt-bHLHPAS, or pBOSGAL4DBD-ArntH378P-bHLHPAS) and the indicated prey vector (50 ng of pBOS or 10, 20, or 50 ng of pBOS3xFLAG-Arnt or pBOS3xFLAG-ArntH378P). After 24 h of culture, the medium was changed, and the cells were further cultured for 24 h. The cell extracts were prepared from the cultured cells and used for luciferase assays. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control.

ever, specifically inhibited its interaction with Arnt2, leading to reduced reporter gene expression. Thus, the PASB domain of AhR determines its binding specificity for Arnt.

We next examined the ability of the His-378 to Pro Arnt mutant to interact with AhR; it behaved virtually identically to Arnt2 (Fig. 6*B*, *columns 7* and *8 versus 10* and *11*), indicating that Pro-352 in Arnt2 is primarily responsible for its reduced affinity for AhR. In these experiments, the prey proteins were expressed almost equally (Fig. 6*C*).

Homodimerization of Arnt and Arnt H378P—Arnt can form a heterodimer with AhR and HIF1α, but it also homodimerizes and binds E-box sequences (25, 34, 35). We investigated the ability of ArntH378P to homodimerize with either WT or mutant Arnt, using the mammalian two-hybrid assay with GAL4DBD-Arnt-bHLHPAS or GAL4DBD-ArntH378P-bHLHPAS as bait (Fig. 3*A*, *constructs 1* and *7*) and a full-length 3xFLAG-Arnt or its H378P mutant as prey (Fig. 5*A*, *constructs 1* and *4*). In any combination of bait and prey, both WT and H378P mutant Arnt activated similar levels of reporter gene expression (Fig. 6*D*), indicating that Arnt homodimerization is not sensitive to the H378P mutation. This result also suggests that Arnt PASB His-to-Pro mutation influences specifically AhR-Arnt dimerization, not the general dimerization.

DISCUSSION

Arnt and Arnt2 are structurally very similar and are believed to be common obligate dimerization partners for a number of bHLH-PAS transcription factors including AhR, HIFα, and Sims (13). We found no differences in the ability of Arnt, Arnt2, or any mutant or chimeric constructs used to activate gene expression in response to hypoxia in both transient and stable expression systems, suggesting that both proteins are equally able to bind HIFα, the key transcription factor mediating the hypoxic response. Thus, Arnt and Arnt2 play functionally interchangeable and overlapping roles in glycolysis, erythropoiesis, and angiogenesis in response to hypoxic conditions. The compensatory effects of Arnt and Arnt2 are noticeable in central nervous system development where Arnt2 is expressed abundantly (36, 37). Whole-mount PECAM (platelet endothelial cell adhesion molecule) immunohistochemistry on the embryonic central nervous system revealed no obvious differences between the WT and Arnt2$^{-/-}$ embryos, whereas Arnt$^{-/-}$ embryos had clear disruptions of the vascular endothelial network (20). This observation suggests that the expression of *VEGF* and other HIFα target genes in Arnt2$^{-/-}$ embryos is effectively normal, presumably because of the compensatory effects of Arnt. In the later stage of central nervous system development, however, several unique functions of Arnt2 become apparent, and Arnt2$^{-/-}$ embryos die perinatally with impaired hypothalamic development (15).

In contrast to their overlapping ability to bind HIFα, and also in apparent contrast with a previous report (13), clear differences were seen in the ability of Arnt and Arnt2 to interact with AhR. XRE-driven luciferase activity in cells expressing Arnt2 was much less than that seen in cells expressing Arnt following

3MC treatment, and the transcription of an XRE-responsive endogenous gene, *CYP1A1*, was completely absent in Hepa1-c4 cells stably expressing Arnt2 after treatment with 3MC (Fig. 4*E*, *columns 4* and *5*). In contrast, Hepa1-c4 cells stably expressing Arnt induced high levels of *CYP1A1* transcripts following 3MC treatment (Fig. 4*E*, columns 2 and 3). Some activation of the XRE-driven reporter gene by Arnt2 (about one-fifth of the Arnt activation) in the previous report (13) is misleading, most probably because of overexpression of Arnt2 in the transient DNA transfection experiments. Arnt2 expressed in these transformant cells was clearly functional, as shown by its ability to induce HRE-responsive genes in conjunction with HIFα (Fig. 4*D*), suggesting that Arnt2 plays little or no role in the cellular response to xenobiotics to induce the XRE-inducible genes in conjunction with AhR. This conclusion is further supported by the observation that zebrafish injected with a zfArnt2 antisense morpholino induced zfCYP1A1 in response to TCDD- as well as sham-treated animals, and there were no differences in TCDD-induced cytotoxicities requiring Arnt/AhR dimerization, such as pericardial edema, reduced trunk blood flow, and shortened lower jaw between these animals (38).

The bHLH domain of AhR is thought to be essential for heterodimer formation between bHLH-PAS transcription factors, and the bHLH domain alone of AhR is capable of dimerizing with the unrelated bHLH-Zip transcription factor USF (39). When the PASA domain is added to the AhR bHLH domain, however, AhR becomes much less promiscuous and stably binds Arnt (39). We showed that a construct consisting of the AhR bHLH and PASA domains binds both Arnt and Arnt2 (Fig. 6*B*, *columns 5* and *11*), but the addition of the PASB domain restricts AhR binding exclusively to Arnt (Fig. 6*B*, *columns 4* and *10*). Taken together, these data indicate that Arnt2 is not likely involved in the induction of drug-metabolizing enzymes following exposure to exogenous aromatic chemicals and other biological processes requiring AhR as a transcription factor.

A recent study reported that both the PASA and PASB domains of HIFα are necessary for heterodimer formation with Arnt (40). The minimal PAS domain structure consists of a 3-stranded β sheet, designated the β-scaffold, and a central α-helical PAS core region containing three short stretches of α-helices and β-sheets connected by a single α-helix called the helical connector. Mutational analysis revealed that hydrophobic interactions between conserved amino acids on the surface of the β-scaffold of HIFα and Arnt are important for heterodimerization, and replacement of hydrophilic amino acids (Q322E, M338E, and Y342T in HIF2α) disrupted this interaction leading to reduced transcription activity in response to hypoxia (40–42). Replacement of His-378 with Pro in Arnt, a residue a little away from the β-scaffold, disrupted its interaction with AhR but had no effect on the interaction of Arnt with HIFα. This suggests that the PASB/PASB-interacting surfaces or modes of AhR and Arnt are different from those of HIFα and Arnt. Additionally, the structural basis for the inhibition of AhR binding by Pro-352 of Arnt2, without affecting HIFα binding, should be examined. Such studies could provide valuable insight into the molecular regulation of the xenobiotic response as well as the diversification of a highly conserved family of transcription factors.

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Effects of coumestrol on lipid and glucose metabolism as a farnesoid X receptor ligand ☆

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ABSTRACT

In the course of an effort to identify novel agonists of the farnesoid X receptor (FXR), coumestrol was determined to be one such ligand. Reporter and *in vitro* coactivator interaction assays revealed that coumestrol bound and activated FXR. Treatment of Hep G2 cells with coumestrol stimulated the expression of FXR target genes, thereby regulating the expression of target genes of the liver X receptor and hepatocyte nuclear factor-4α. Through these actions, coumestrol is expected to exert beneficial effects on lipid and glucose metabolism.

The farnesoid X receptor (FXR; NR1H4) is a member of a nuclear receptor superfamily expressed predominantly in the intestine, kidney, and liver. Upon activation by bile acids, which are endogenous FXR ligands, FXR regulates the expression of genes involved in bile acid homeostasis, such as cholesterol-7α hydroxylase [1] and ileal bile acid binding protein [2]. The identification of phospholipid transfer protein (PLTP) [3] and apolipoprotein A-I [4] as FXR target genes suggests that FXR also controls triglyceride (TG) metabolism. Recent studies using FXR-deficient mice, in which serum glucose levels are elevated, demonstrated that FXR also has a regulatory role in glucose homeostasis [5]. In addition, FXR activation induces the expression of the small heterodimer partner (SHP; NR0B2) [6,7], which acts as a corepressor for several nuclear receptors, including liver X receptor α (LXRα; NR1H3) and hepatocyte nuclear factor-4α (HNF-4α; NR2A1) [8,9]. LXRα is a sterol-responsive transcription factor that regulates the expression of sterol response element-binding protein 1c (SREBP1c) [10,11], which in turn regulates lipogenic enzymes. The best-characterized HNF-4α target genes are those involved in lipid transport and glucose metabolism. Thus, FXR regulates lipid and glucose metabolism through the FXR-SHP cascade.

In general, nuclear receptors regulate gene expression in response to ligand binding through the direct recruitment of cofactors [12–14]. In the presence of agonists, the receptor binds coactivators (e.g., TIF2), and in turn associates with additional proteins to form a complex that activates transcription [15]. In the ab-

Abbreviations: ABCA1, ATP-binding cassette transporter A1; AP, alkaline phosphatase; apoB, apolipoprotein B; apoC-III, apolipoprotein C-III; BAP, bacterial alkaline phosphatase; CDCA, chenodeoxy cholic acid; CoA-BAP, coactivator-bacterial alkaline phosphatase; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; FAS, fatty acid synthase; FXR, farnesoid X receptor; GST, glutathione-*S* transferase; HNF-4α, hepatocyte nuclear factor-4 α; LBD, ligand binding domain; LXRα, liver X receptor α; MTP, microsomal triglyceride transfer protein; NID, nuclear receptor interaction domain; NR, nuclear receptor; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; PLTP, phospholipid transfer protein; RT-PCR, reverse-transcription polymerase chain reaction; SHP, small heterodimer partner; SREBP1c, sterol response element-binding protein 1c; TG, triglyceride.

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sence of agonists, nuclear receptors bind corepressors (e.g., NCoR) and form a complex that represses transcription [16]. The coactivator-bacterial alkaline phosphatase (CoA-BAP) system developed by Kanayama et al. is a new cell-free assay system for evaluating ligand activity that is based on the ligand-dependent interactions between nuclear receptors and cofactors [17]. Using this system to screen food compounds and their derivatives, we determined that coumestrol is a potent FXR agonist. We show here that coumestrol activates FXR, and thereby regulates the expression of genes involved in lipid and glucose metabolism.

Materials and methods

Materials. Coumestrol, chenodeoxy cholic acid (CDCA), and 17-β-estradiol were purchased from Sigma–Aldrich Co. (St. Louis, MO). An FXR ligand, GW4064, was custom synthesized.

CoA-BAP system. LBD protein (ligand binding domain of nuclear receptors) fused to glutathione-*S* transferase (GST) and NID protein (nuclear receptor interaction domain of cofactors) fused to BAP were prepared as described previously [17]. The purified GST-hFXR LBD protein sample, diluted in 0.1 M $NaHCO_3$ (pH 8.4), was incubated on 96-well plates (MaxiSorp; Nalge Nunc Internationals, Roskilde, Denmark). After overnight incubation at 4 °C, excess protein was removed. An appropriate concentration of a test chemical was then added. After 1 h of incubation at 4 °C, excess test chemical was aspirated and purified hTIF2 NID-BAP fusion protein was applied. After 1 h incubation at 4 °C and subsequent washing, *p*-nitrophenyl-phosphoric acid was added to each well as a BAP substrate, and the enzyme reaction was performed at 30 °C. After an appropriate incubation period, the alkaline phosphatase (AP) activity was measured spectrophotometrically at 405 nm on a microtiter plate reader.

Cell culture. The human hepatoma cell line Hep G2 and the embryonic kidney cell line HEK293T were cultured as previously described [18]. The breast cancer cell line MCF-7 was maintained in minimal essential medium (MP Biomedicals, Irvine, CA) supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Reporter assays. The expression plasmids pGAL4-hFXR and pFLAG-hFXR [19] and reporter plasmid pSHPp-Luc [18] were previously described. HEK293T cells (12-well plate) were transfected with 300 ng of a reporter plasmid (pUAS-Luc or pSHPp-Luc) and 100 ng of a hFXR expression plasmid (pGAL4-hFXR or pFLAG-hFXR), together with 100 ng of a SV40-gal internal control plasmid using the calcium phosphate transfection method. Twenty-four hours after transfection, the cells were treated with test chemicals in minimal essential medium (phenol red free) supplemented with 5% charcoal-stripped fetal bovine serum. After 24 h exposure to the chemicals, luciferase activities were quantified using a Luciferase Assay Systems (Promega, Madison, WI) according to the manufacturer's instructions. β-Galactosidase activity was used as an internal control.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from Hep G2 cells or MCF-7 cells using an RNA extraction reagent (ISOGEN; Nippon Gene, Toyama, Japan) and reverse-transcribed using TaqMan reverse transcriptase reagents (Applied Biosystems, Weiterstadt, Germany). Fluorescent real-time PCR was performed using SYBR green PCR master mix on an ABI 7700 system. The sequences of the primers used for quantitative RT-PCR are shown in Supplementary Information Table 1. The mRNA expression data were normalized to 36B4 mRNA levels. TaqMan Gene Expression Assays were used for pS2/TFF1 (Applied Biosystems).

ApoB ELISA assay. Hep G2 cells were cultured in medium containing 5% lipoprotein-deficient serum and treated with test chemicals for 3d. At 18 h before the end of the 3d treatment, the media were replaced with serum-free media. At the end of the treatment the media were collected and apoB secretion was measured using an ApoB Microwell ELISA Assay Kit (ALerCHEK Inc., Portland, ME) according to the manufacturer's instructions. ApoB secretion was normalized by cell protein determined using a bicinchoninic acid assay (Pierce Chemical Co., Millford, IL).

Results

Ligand-dependent interaction between nuclear receptor and cofactor

To assess the FXR agonist activity of the test compounds, we used an in vitro coactivator interaction assay, the CoA-BAP system. We confirmed that a natural FXR ligand, CDCA, and a synthetic ligand, GW4064, enhanced the AP activity in a dose-dependent manner. Coumestrol increased the AP activity in the FXR-TIF2 system (Fig. 1). In the absence of FXR LBD (GST alone), there was no apparent increase in the AP activity.

Effects of coumestrol on transcriptional activity of FXR

To investigate the effect of coumestrol on FXR transcriptional activity, we used a GAL4 system using a fusion protein that included a GAL4 DNA-binding domain and a full-length hFXR. Micromolar concentrations of coumestrol increased the reporter activity dose-dependently, while CDCA (100 μM) and GW4064 (0.1 μM) caused approximately 30- and 80-fold inductions, respectively (Fig. 2A).

To further examine the effect of coumestrol on FXR transcriptional activity, a reporter assay was performed using the human SHP promoter-containing reporter gene. The reporter activity was induced by 50 μM coumestrol to 2.0-fold over that in non-treated cells, while CDCA (100 μM) and GW4064 (1 μM) enhanced the reporter activity 4- and 3-fold, respectively (Fig. 2B).

Effects of coumestrol on the expression of genes involved in lipid and glucose metabolism

Although the GAL4 system in Fig. 2A revealed that coumestrol is a less potent ligand than CDCA, another reporter assay in Fig. 2B showed comparable effects between them. To evaluate the effect of coumestrol on lipid and glucose metabolism, we performed

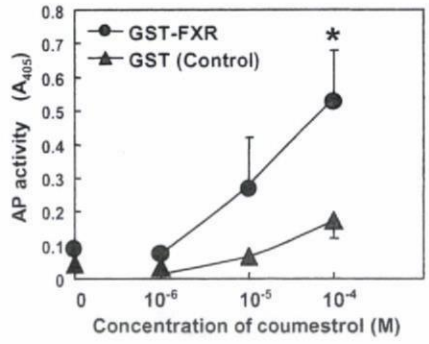

Fig. 1. Assessment of FXR ligand activity using a CoA-BAP system. The CoA-BAP assay was performed with increasing amounts of coumestrol, as described in the Materials and methods. Ligand-dependent interactions between GST-hFXR LBD and hTIF2 NID-BAP were determined as AP activity. AP activity without GST fusion protein was measured as background. Means ± SD ($n = 3$) are shown. $^*P < 0.05$ compared with control (GST alone).

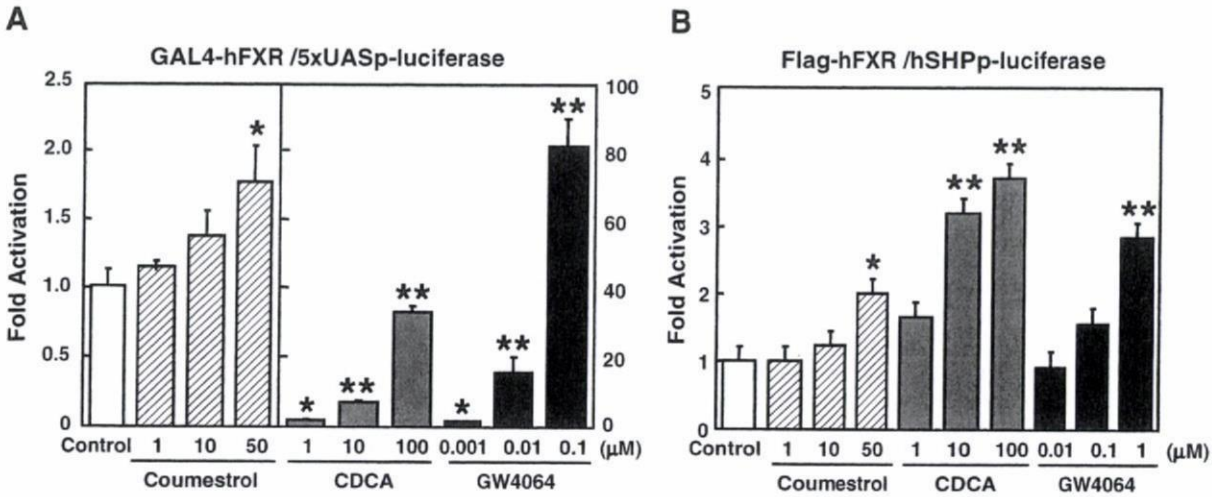

Fig. 2. Coumestrol stimulates FXR transcriptional activity. HEK293T cells were cotransfected with a pGAL4-hFXR expression plasmid and a pUAS-Luc reporter plasmid together with a SV40-gal internal control plasmid and treated for 24 h with coumestrol (1, 10, or 50 μM), CDCA (1, 10, or 100 μM), GW4064 (0.001, 0.01, or 0.1 μM), or DMSO (control) (A). HEK293T cells were cotransfected with a pFLAG-hFXR expression plasmid and a reporter plasmid of SHPp-Luc, together with a SV40-gal internal control plasmid, and treated with coumestrol (1, 10, or 50 μM), CDCA (1, 10, or 100 μM), GW4064 (0.01, 0.1, or 1 μM), or DMSO (control) (B) for 24 h before assaying luciferase activity. Means ± SD ($n = 3$) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared vehicle-treated cells.

quantitative RT-PCR to measure the mRNA levels of metabolism-related genes. The expression levels of FXR target genes, such as SHP and PLTP, were upregulated in Hep G2 cells treated with coumestrol as well as CDCA for 24 h (Fig. 3A), suggesting that the GAL4 system in Fig. 2A underestimated coumestrol functions. Coumestrol treatment decreased the expression of LXR target genes, such as SREBP1c and ATP-binding cassette transporter A1 (ABCA1), SREBP1c target fatty acid synthase (FAS) (Fig. 3B), and HNF-4α target genes, such as microsomal triglyceride transfer protein (MTP), apolipoprotein B (apoB), apolipoprotein C-III (apoC-III), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) (Fig. 3C). To exclude the possibility that the upregulation of SHP by coumestrol was accomplished via the estrogen receptor (ER) [20], we evaluated the estrogen responsiveness of Hep G2 cells. In ER-positive MCF-7 cells treated with 10 nM 17-β-estradiol, expression of the ER target gene pS2/TFF1 increased over 14-fold compared to untreated controls, whereas neither pS2/TFF1 nor SHP were induced by 17-β-estradiol in Hep G2 cells (data not shown).

Reduced apolipoprotein B secretion from Hep G2 cells

To examine whether the suppression of MTP and apoB gene expression leads to reduced apoB secretion, we determined the

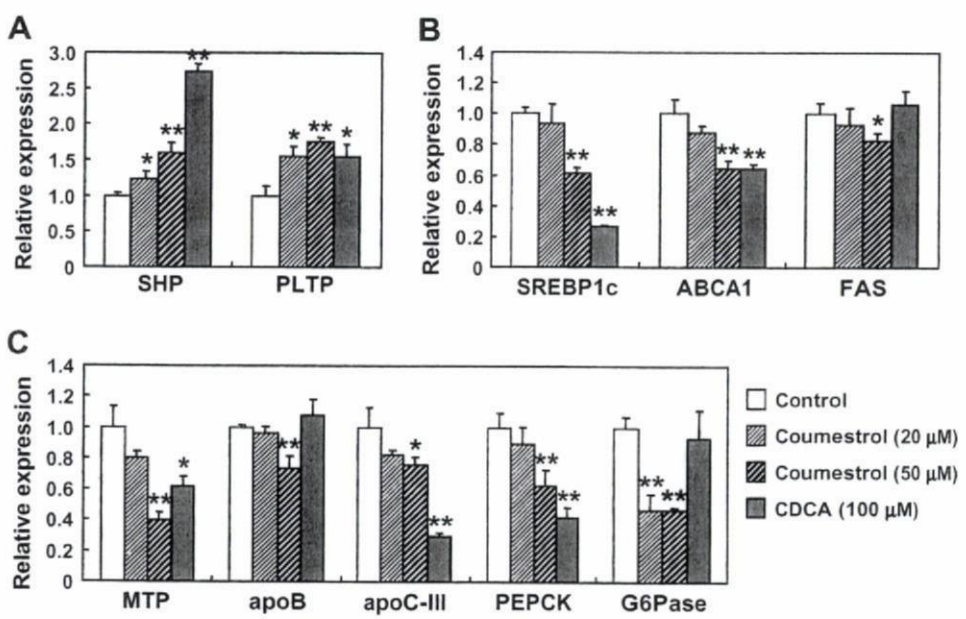

Fig. 3. Coumestrol regulates the expression of genes that are involved in lipid and glucose metabolism. Hep G2 cells were incubated with DMSO (control), coumestrol (20 or 50 μM), or CDCA (100 μM) for 24 h. Hepatic expression levels of FXR target genes (SHP and PLTP) (A), LXR target genes (SREBP1c, ABCA1, and FAS) (B), and HNF-4 target genes (MTP, apoB, apoC-III, PEPCK and G6Pase) (C) were measured using quantitative RT-PCR. Data are presented as the fold-change in gene expression relative to that in DMSO-treated control cells. Means ± SD ($n = 3$) are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated cells.

levels of apoB secreted in the culture medium of Hep G2 cells incubated with coumestrol. Coumestrol (50 μM) reduced apoB secretion by 50% compared to the control, comparable to the decrease observed with CDCA (Fig. 4). Although CDCA treatment had no significant effect on apoB gene expression (Fig. 3C), it seems likely that MTP activity should be a determinant for apoB-containing lipoprotein secretion.

Discussion

Coumestrol is a coumestan phytoestrogen present in soy sprouts and alfalfa [21]. Previous studies demonstrated that coumestrol binds ERα and ERβ [22], effectively lowers total serum cholesterol in ovariectomized rats [23], and prevents ovariectomy-induced bone loss [24]. These effects of coumestrol are generally considered to be due to its estrogenic activity. ERα directly binds the SHP promoter and enhances transcription [20]. This raises the possibility that coumestrol enhanced SHP gene expression in Hep G2 cells through activation of ERα in Fig. 3. Consistent with the previous finding that Hep G2 cells lack any ER activities [20], 17-β-estradiol stimulated the expression of pS2/TFF1, an ER target gene, only in the ER-positive cell line MCF-7, but not in Hep G2 cells. These findings indicate that coumestrol increases SHP gene expression through the direct activation of FXR.

The present study shows a novel regulatory mechanism by which coumestrol exerts its beneficial effects through FXR activation. Reporter gene and *in vitro* coactivator interaction assays revealed that this compound is a potent FXR agonist, thereby bringing about the increased expression of FXR target genes. The subsequent increase in SHP suppresses the expression of LXR and HNF-4α target genes. At the cellular level, coumestrol decreased apoB secretion, as reflected by the downregulation of MTP and apoB gene expression. These results indicate that coumestrol exerts various effects as a bifunctional ligand via an FXR-mediated pathway as well as through an ER-mediated pathway.

In the current experiments, high levels of coumestrol concentration (~50 μM) were required to exert significant effects resembling endogenous FXR ligand, CDCA (100 μM). However, given that both of them were effective at the comparable concentration, coumestrol is likely to be one of potent FXR ligands. Cholic acid, another endogenous FXR ligand, prevents hepatic TG accumulation, very-low density lipoprotein secretion, and elevated serum TG in mouse models of hypertriglyceridemia [25]. Recent *in vivo* studies also demonstrate that FXR ligands are potentially useful targets for the treatment of cholestatic liver disease [6] and dyslipidemia [26]. Taken together, our findings suggest that coumestrol might improve metabolic disorders such as hypertriglyceridemia, fatty liver disease and diabetes, at least partly through the activation of FXR.

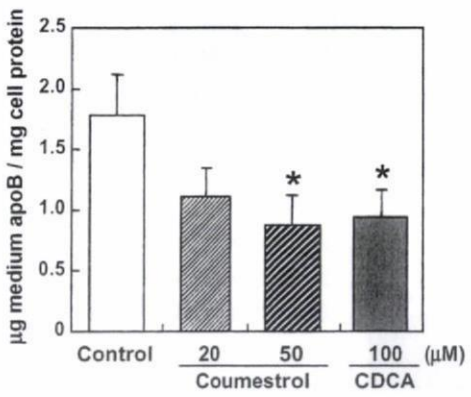

Fig. 4. Coumestrol reduced apoB secretion from Hep G2 cells. Hep G2 cells were cultured in medium containing DMSO (control), coumestrol (20 or 50 μM), or CDCA (100 μM) for 3 d, and apoB secretion into media for the last 18 h was measured using ELISA. Means ± SD ($n = 3$) are shown. $^{*}P < 0.05$ compared with vehicle-treated cells.

A recent report demonstrated that coumestrol functions as a naturally occurring agonist of the pregnane X receptor [27]. In the report, this compound was evaluated as a week agonist of several nuclear receptors including FXR using a GAL4 system that must be a similar system we used. It is possible that coumestrol greatly regulated gene expression of FAS, apoB and G6Pase rather than CDCA (Fig. 3) by taking advantage of the ligand activities on multiple nuclear receptors.

In conclusion, the results of the present study indicate that coumestrol activates FXR and regulates the expression of genes involved in lipid and glucose metabolism, suggesting that coumestrol exerts beneficial effects on metabolic disorders, including hypertriglyceridemia and diabetes. Moreover, the results provide evidence that the CoA-BAP system, in combination with various other analyses, is a practical and useful new strategy for identifying novel bioactive substances.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.04.136.

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Screening and detection of the *in vitro* agonistic activity of xenobiotics on the retinoic acid receptor

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Abstract

The retinoic acid receptors (RARs) play key roles in various biological processes in response to endogenous retinoic acids. However, excessive embryonic exposure to specific ligands for each subtype of the RAR was reported to induce specific developmental abnormalities. We measured the RAR agonistic activity of 543 chemicals using an assay system adopting yeast cells transfected with the human RARγ and a coactivator. Eighty-five of the 543 chemicals, including 16 organochlorine pesticides, 14 styrene dimers, 9 monoalkylphenols and 6 parabens, exhibited RARγ agonistic effects in this assay. In particular, monoalkylphenols having a 6–9 carbon alkyl group *para* to the phenolic hydroxyl group possessed high affinity for the RARγ, and their activities were 1.363–0.446% of that of *all-trans* RA. *para*-Alkylphenols chlorinated at the *ortho* position also were about as active or more active than their unchlorinated analogs. In addition, all tested styrene dimers showed positive effects, and the activity of 1-phenyltetralin, the strongest in this category, was 1.169% that of *all-trans* RA. A number of chemicals having binding affinity for the RARγ were revealed in this study (both newly identified and confirmed), further comprehensive studies of *in vitro* and *in vivo* effects via the RARs are required for the reliable risk assessment of chemicals. *In vitro* receptor binding studies represent an important step in hazard identification and suggest a potential mechanism of action, which can be an important step in risk assessment and in particular for screening studies to identify potential toxicity and inform mechanistic studies.

Keywords: Retinoic acid receptor; Two-hybrid yeast assay; Monoalkylphenol; Styrene dimer; Paraben; Organochlorine pesticide

1. Introduction

The retinoic acid (RA) receptors (RARs) are, like the steroid hormone and thyroid hormone receptors (TR), nuclear receptors that respond to specific natural ligands, *all-trans* RA and 9-*cis* RA. Both RAs, which are oxidative metabolites of vitamin A, are essential for cellular proliferation, development and differentiation in vertebrates and therefore play crucial roles in normal growth and homeostasis. However, both deficiency and excess of vitamin A are harmful. Vitamin A deficiency during gestation results in diverse embryonic malformations in various organs (Zile, 1998, 2001), while excess RA has been reported to trigger teratogenic actions in the developing embryo via the RARs. Ligands specific for each subtype of the RARs (α, β and γ) have been reported to induce specific deformities in rodent embryos (Elmazar et al., 1996, 2001). A ligand for the α-subtype causes defects of the ear, mandible and limb, a β-subtype ligand causes defects of the urinary system and liver, and a γ-subtype ligand causes ossification deficiencies and defects of the sternebrae and vertebral body.

We have developed several yeast two-hybrid systems transduced with the ligand binding domains of nuclear receptors and a coactivator for the receptors for detecting and measuring the activity of chemicals (Nishikawa et al., 1999; Shiraishi et al., 2000). In prior works, it has been found that there are a number of industrial/environmental

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compounds with the capability to activate or inactivate nuclear receptors such as the estrogen receptors (ERs) and the TRs, and some compounds show unexpected activity; i.e., activity that would not be readily predicted from the structure of the compounds (Shiraishi et al., 2003; Arulmozhiraja et al., 2005; Morohoshi et al., 2005). These studies indicated that chemicals encountered in daily life or through accidental exposure bind with nuclear receptors and suggested potential toxicity, mechanisms of action and therefore potential risks to human health. In our laboratory, yeast assays with nuclear receptors derived from the Japanese medaka fish (*Oryzias latipes*) as well as the human have also demonstrated the receptor activation of environmental water samples taken from contaminated rivers, lakes and seas (Mispagel et al., 2005; unpublished data, Shiraishi et al., 2006) and thereby show that organisms in the environment may be exposed to potentially harmful chemicals and/or bioactive substances originating from human activity.

The objective of the present study was to screen a wide range of xenobiotic and other compounds for agonistic effects on the RAR and to quantify their activities. We prepared a yeast two-hybrid system to detect transcriptional activation via the human RARγ and assessed 543 compounds including industrial chemicals, agrochemicals, natural compounds, medicines and cosmetic chemicals. An assay for each human RAR subtype was derived from previously reported yeast assays and optimized for high-throughput screening. Because the yeast cells transfected with the γ-subtype showed the lowest luminescence intensity when inactivated and the highest reactivity to an endogenous ligand, *all-trans* RA, we selected the RARγ type of assay for the present investigation. As the RAR agonistic effect was detected in different categories of chemicals, we provide the measured activities of the positively-reacting compounds grouped according to chemical structure.

2. Materials and methods

2.1. Compounds

The 543 compounds examined in this study are listed in Table 1, grouped according to their intended uses and chemical structures. The compounds were purchased from Accu Standard, Inc. (New Haven, CT, USA), Acros Organics N.V. (Geel, Belgium), Alfa Aesar GmbH & Co., KG (Karlsruhe, Germany), Cosmo Bio Co., Ltd. (Tokyo, Japan), Dr. Ehrenstorfer GmbH (Augsburg, Germany), GL Science, Inc. (Tokyo, Japan), Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Katayama Chemical Industries Co., Ltd. (Osaka Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Maruzen Petrochemical Co., Ltd. (Tokyo, Japan), MP Biochemicals (Solon, OH, USA), PerkinElmer, Inc. (Wellesley, MA, USA), Scientific Polymer Products, Inc. (Ontario, NY, USA), Sigma–Aldrich Corp. (St. Louis, MO, USA), Steraloids, Inc. (Newport, RI, USA), Tocris Bioscience (Ellisville, MO, USA), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Toronto Research Chemicals Inc. (North York, ON, Canada) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or were gifts from researchers who had synthesized them for other purposes.

2.2. Yeast two-hybrid assay

The transcriptional agonistic activities of compounds to the RAR were measured with a reporter assay using yeast cells (*Saccharomyces cervisiae* Y190). An expression plasmid for the ligand binding domain of the human RARγ and the coactivator pGAAD424-TIF-2 was introduced into yeast cells that carried the β-galactosidase reporter gene (Nishikawa et al., 1999). The assay was performed using a chemiluminescent reporter gene method (for β-galactosidase) employing a 96-well culture plate, based on a yeast two-hybrid estrogenicity assay (Shiraishi et al., 2000, 2003).

Yeast cells were preincubated for 24 h at 30 °C with shaking in modified SD medium lacking tryptophan and leucine (5.8% yeast nitrogen base, 0.75% dextrose, 0.013% L-valine, 0.00435% L-phenylalanine, 0.00261% L-isoleucine, L-lysine HCl and L-tyrosine, 0.00174% L-adenine hemisulfate salt, L-arginine HCl, L-histidine HCl monohydrate, L-methionine and L-uracil) and the cell density was adjusted to an absorbance of 1.65–1.80 at 595 nm. A dimethylsulfoxide (DMSO) solution of each test compound was stored at −80 °C until just before examination and was serially twofold diluted with the medium. An aliquot of the diluted solution (120 μl) was poured to two wells of a black 96-well culture plate for chemiluminescence measurement, and then the yeast cell suspension (60 μl) was added. At least seven serial two-fold concentrations of each chemical from 10 μM to 156 nM were tested; lower concentrations were tested for chemicals showing high RAR agonistic activity. The solution in every well contained 1% DMSO. After vortex mixing, the plate was incubated at 30 °C under conditions of high humidity for 4 h. A solution (80 μl) for inducing chemiluminescence from released β-galactosidase, consisting of reaction buffer (30 μl) containing GalactLux substrate (AURORA GAL-XE, MP Biochemicals) and a 1:1 mixture (50 μl) of zymolyase 20 T and 100 T solutions for enzymatic digestion (Kirin Brewery Co, Ltd., Tokyo, Japan), was added to each well. The plate was incubated at 37 °C for 1 h and then placed in a 96-well plate luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan), and a light emission accelerator solution (AURORA GAL-XE, 50 μl) was added to each well using the luminometer pump. The chemiluminescence produced by released β-galactosidase in each well was measured.

All test compounds were evaluated in a minimum of three separate assays which were performed in duplicate. For comparative estimates of the ability of test compounds to activate the RAR, *all-trans* RA, an endogenous agonist

Table 1
List of 543 compounds tested in a yeast two-hybrid assay for the RARγ

Compounds
Industrial chemicals (252)
Aromatic hydrocarbons (10)
Biphenyl
2-Terphenyl
3-Terphenyl
4-Terphenyl
n-Butylbenzene
n-Octylbenzene
n-Nonylbenzene
1,3-Diphenylpropane
Benzylbiphenyl
Triphenylmethane
Polycyclic aromatic hydrocarbons (14)
Naphthalene
Acenaphthene
Fluorene
Phenanthrene
Anthracene
Pyrene
Fluoranthene
Chrysene
Benz[a]anthracene
Benzo[a]pyrene
Benzo[e]pyrene
Benzo(k)fluoranthene
Benzo(b)fluoranthene
Dibenz(a,h)anthracene
Arsenic compounds (13)
Sodium meta-arsenite
Dimethyl arsinic acid
Methyl arsonic acid
Arsenic(III)oxide
Phenyldimethylarsine oxide
Phenylmethylarisine oxide
Phenyl arsonic acid
Diphenylmethylarsine oxide
Diphenyl arsinic acid
Triphenyl arsine
Sodium arsenate (dibasic) 7H2O
Triphenyl arsine oxide
10,10'-Oxybis(phenoxyarsine)
Bisphenols and related chemicals (37)
Octafluoro-4,4'-biphenol
2,2',6,6'-TetramethylbisphenolA
4,4'-Thiodiphenol
Bisphenol A
2,2',6,6'-Tetrabromobisphenol A
2,2',6,6'-Tetrachlorobisphenol A
Bisphenol B
2-Chlorobisphenol A
2,2'-Dichlorobisphenol A
2,6-Dichlorobisphenol A
2,2',6-Trichlorobisphenol A
4,4'-Methylenebisphenol (Bisphenol F)
4,4'-Sulfonyldiphenol (Bisphenol S)
3,4-(1-Methyl-1-phenylethyl)diphenol
2,4'-Isopropylidenediphenol
4,4'-(1,3-Dimethylbutylidene)bisphenol
Bisphenol E
2,4'-Dihydroxydiphenyl sulfone (Bisphenol S iso.)
2,2'-Isopropylidenediphenol
4,4'-Cyclohexyliden bisphenol
4,4'-(1-Ethyl-2-methyltrimethylene)bisphenol
4,4'-Diisobutylethylidenediphenol
4-Hydroxyacetophenone
4-Hydroxy-4'-isopropoxy diphenylsulfone
4,4'-Isopropoxy diphenylsulfone
4-[1-(4-Hydroxyphenyl)-1-methylethyl]-1,2-benzoquinone
Bisphenols and related chemicals (Continued)
4-Hydroxyphenyl isopropanol
4-Hydroxyphenyl isobutyl methyl ketone
6-Hydroxy-1-(4-hydroxyphenyl)-1,3,3-trimethylindane
4-Cumylphenol
2-(4-Hydroxyphenyl)-2,4,4-trimethylchroman
6-Hydroxy-3-(4-hydroxyphenyl)-1,1,3-trimethylindane
2,4-Bis(4-hydroxycumyl)phenol
4-(4-Hydroxyphenyl)-2,2,4-trimethylchroman
4-Propenylphenol
4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene
2-(2-Hydroxyphenyl)-2,4,4-trimethylchroman
Dyes (21)
Azobenzene
N-Acetyl-5-hydroxy-tryptamine
Acid Alizarin Violet N
1-Acetyl-4-(4-hydroxyphenyl)-piperazine
Anthrarobin
Anthraflavic acid
cis-1,3-O-Benzylideneglycerol
Chlorophenol Red
2,5-Dihydroxyphenylacetic γ-lactone
4-(2,4-Dinitroanilino)-phenol
6-Fluoro-4-hydroxy-coumarin
Hoechst 33258
6-Hydroxy-1,3-benzoxathiol-2-one
2-Hydroxy-9-fluorenone
4-Hydroxyindole
4-Hydroxy-6-methyl-2-pyrone
3-Hydroxy-1H-phenalen-1-one
2-(4-Hydroxyphenyl)-5-pyrimidinol
1-Hydroxypyrene
Indigo, carmine
Indigo, synthetic
Metals (5)
Tributyltin(IV)chloride
Triphenyltin(IV)chloride
Dibutyltin(IV)dichloride
Lead (II) chloride
Lead (II) acetate trihydrate
Monoalkyl phenols and related chemicals (27)
2-n-Propylphenol
3-n-Propylphenol
4-n-Propylphenol
2-Isopropylphenol
3-Isopropylphenol
4-Isopropylphenol
4-n-Butylphenol
2-s-Butylphenol
4-s-Butylphenol
2-t-Butylphenol
3-t-Butylphenol
4-t-Butylphenol
4-n-Pentylphenol
4-t-Pentylphenol
4-n-Hexylphenol
4-n-Heptylphenol
4-n-Octylphenol
4-t-Octylphenol
4-n-Nonylphenol
4-Nonylphenol (mixed isomers)
4-Dodecylphenol (mixed isomers)
2-Chloro-4-butylphenol
2,6-Dichloro-4-butylphenol
2-Chloro-4-octylphenol
2,6-Dichloro-4-octylphenol
2-Chloro-4-nonylphenol
2,6-Dichloro-4-nonylphenol
Parabens (13)
3-Hydroxybenzoic acid
4-Hydroxybenzoic acid
Methyl 4-hydroxybenzoate
Ethyl 4-hydroxybenzoate
Propyl 4-hydroxybenzoate
Isopropyl 4-hydroxybenzoate
Butyl 4-hydroxybenzoate
Isobutyl 4-hydroxybenzoate
n-Amyl 4-hydroxybenzoate
n-Hexyl 4-hydroxybenzoate
Benzyl 4-hydroxybenzoate
2-Ethylhexyl 4-hydroxybenzoate
n-Dodecyl 4-hydroxybenzoate
Phenols and related chemicals (37)
4-Cyanophenol
4-Hydroxybenzaldehyde
4-Methoxyphenol
2-Cyclopentylphenol
4-Cyclopentylphenol
2,4-Dichlorophenol
3,4-Dichlorophenol
4-Hydroxycinnamic acid
2-Hydroxybiphenyl
4-Hydroxybiphenyl
4-Cyclohexylphenol
4-Benzylphenol
4-Hydroxybezophenone
Phenyl salicylate
2-Iodophenol
4-Iodophenol
1,1-Bis(4-hydroxyphenyl)-propane
4-(1-Adamantyl)phenol
2-(1-Adamantyl)-4-methylphenol
2,4-Dibromophenol
2,6-Dibromophenol
3-(4-Hydroxyphenyl)propionic acid N-hydroxysuccinimide ester
Hexestrol
4,4-Bis-(4-hydroxyphenyl)valeric acid
Phenolphthalein
4,4'-(1,3-Adamantane-diyl)diphenol
2,4,6-Tribromophenol
3,5-Diiodosalicylic acid
Butylphenoxy acetic acid
2,3-Dichlorophenoxy acetic acid
2-Chloro-4-butylphenoxy acetic acid
4-Nonylphenyl 2-hydroxyethyl ether
2,6-Dichloro-4-butylphenoxy acetic acid
2-Chloro-4-nonylphenyl 2-hydroxyethyl ether
2-Chloro-4-octylphenoxy acetic acid
4-Nonylphenyl 2-(2hydroxy-ethyloxy)ethyl ether
2,6-Dichloro-4-nonylphenyl 2-hydroxyethyl ether
Phthalates (9)
Diethyl phthalate
Di-n-propyl phthalate
Di-n-butyl phthalate
Di-n-pentyl phthalate
Benzyl n-butyl phthalate
Dicyclohexyl phthalate
Di-n-hexyl phthalate
Dibenzyl terephthalate
Di-2-ethylhexyl phthalate

Table 1 (*continued*)

Compounds		
Industrial chemicals (Continued)	Agrochemicals (124)	Organophosphates (Continued)
Styrene dimers (14+1)	Amides (2)	EPN
1,4-Diphenyl-2-butene	Mepronil	Ethion
1-Methyl-1-phenylindan	Flutolanil	Glyphosate
1-Methyl-3-phenylindan		Iprobenfos
1-Phenyltetralin	Benzimidazoles (2)	Isofenphos
2,3-Diphenyl-1-butene	Carbendazim	Isofenphos oxon
2,4-Diphenyl-1-butene	Thiabendazole	Isoxathion
cis-1,4-Diphenyl-1-butene		Isoxathion oxon
trans-1,4-Diphenyl-1-butene	Carbamates (12)	Leptophos
cis-2,4-Diphenyl-2-butene	Aldicarb	Malathion
trans-2,4-Diphenyl-2-butene	Benfuracarb	MEP oxon
cis-1,2-Diphenylcyclobutane	Benomyl	Parathion
trans-1,2-Diphenylcyclobutane	Carbaryl (NAC)	Phenthoate
trans-1,3-Diphenyl-1-butene	Mancozeb	Piperophos
trans-1,3-Diphenylcyclobutane	Maneb	Prothiophos
Polystyrene standard (mixed styrene dimer isomers)	Methomyl	Thenylchlor
	Metiram	Tolclofos-methyl
Styrene trimer (16)	Molinate	
1,2,4-Triphenylcyclohexane	Thiobencarb	Pyrethroids (16)
1,3,5-Triphenyl-1-hexene	Zineb	Allethrin
1,3,5-Triphenylcyclohexane	Ziram	cis-Permethrin
1,4,5-Triphenyl-1-hexene		Cycloprothrin
1a-Phenyl-4a-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Diphenyl ethers (7)	Cyfluthrin
1a-Phenyl-4a-(2-phenylethyl)tetralin	Acifluorfen	Cyhalothrin
1a-Phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Aclonifen	Cypermethrin
1e,2e,4a-Triphenylcyclohexane	Bifenox	Esfenvalerate
1e-Phenyl-4a-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	Chlomethoxynil	Ethofenprox
1e-Phenyl-4a-(2-phenylethyl)tetralin	Chlornitrofen (CNP)	Fenpropathrin
1e-Phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthanene	CNP-amino	Fenvalerate
1-Methyl-1,2,4-triphenylcyclopentane	Nitrofen	Flucythrinate
1-Methyl-1,3,4-triphenylcyclopentane		Fluvalinate
1-Methyl-3-phenyl-2-(1-phenylethyl)indan	Organochlorines (30)	Permethrin
2,4,6-Triphenyl-1-hexene	Aldrin	Resmethrin
2,4,6-Triphenyl-2-hexene	β-Benzene hexachloride (β-BHC)	Tralomethrin
	Chlordane	trans-Permethrin
Others (35)	cis-Chlordane	
4-Nitrotoluene	trans-Chlordane	Triazines (4)
1-Naphthol	Chlordecone	Atrazine
2-Naphthol	o,p'-DDD	Dimethametryn
4-Hydroxy-1-indanone	o,p'-DDE	Metribuzin
5,6,7,8-Tetrahydro-1-naphthol	o,p'-DDT	Simazine (CAT)
5,6,7,8-Tetrahydro-2-naphthol	p,p'-DDD	
Sodium pyrithione	p,p'-DDE	Ureas (2)
1,2-Benzothiazol-3-one	p,p'-DDT	Diuron
Biphenyl ether	Dicofol	Pencycuron
2-n-Octyl-4-isothiazolin-3-one	Dieldrin	
N,N',N''-Trishydroxyethylhexahydro-s-triazine	α-Endosulfan	Others (22)
Benzyl-2-naphthylether	β-Endosulfan	Alachlor
4-Nonylcatechol	Endrin	Amitrole
1-(4-Hydroxyphenyl)-1-nonanol	Fthalide	Bromofenoxim
Methyl o-benzoylbenzoate	Heptachlor	Buprofezin
1,2-Bis(3-methylphenoxy)ethane	cis-Heptachlor epoxide	Captan
Triphenylborane	trans-Heptachlor epoxide	Chlorothalonil
1-Nitropyrene	Hexachlorobenzene	Coumachlor
2-Iodobenzoic Acid	1,2,3,4,5,6-Hexachlorocyclohexane (γ-BHC)	Dazomet
Dipyrithione	Methoxychlor	1,2-Dibromo-3-chloropropane (DBCP)
6-Bromoharman	Mirex	Dichlofluanid
Phenyl-1-hydroxy-2-naphthoate	trans-Nonachlor	(2,4-Dichlorophenoxy)acetic acid
β-Naphthoflavone	Oxychlordane	2-(3-Chlorophenoxy)-propionic Acid
Zinc pyrithione	Pentachlorophenol (PCP)	1,3-Dichloropropene
Triphenylborane-pyridine complex	Toxaphene	Fluazifop-butyl
6,8-Dibromoharman	2,4,5-Trichlorophenoxyacetic Acid	Oxadiazon
meso-Stilbene dibromide		Pendimethalin
Copper quinolate	Organophosphates (27)	Probenazole
Di(2-ethylhexyl)adipate	Bromophos-ethyl	Pyroquilon
Octachlorostyrene	Bromopropylate	Tebuconazole
3,6,8-Tribromoharman	Butamifos	Tebufenozide
Triphenylborane-octadecylamin complex	Chlorpyrifos	Trifluralin
Perfluorobutansulfonate (potassium salt)	Chlorpyrifosmethyl	Vinclozolin
Chlorhexidine Hydrochloride	Cyanofenphos	
1,2,5,6,9,10-Hexabromocyclododecane	Diazinon	
	Diazinon oxon	
	Dichlofenthion	
	Edifenphos	

(*continued on next page*)

Table 1 (*continued*)

Compounds		
Natural compounds and related chemicals (109)	Natural compounds (continued)	Medicines and cosmetic-related chemicals (58)
Natural compounds (72)	Melatonin	Medicines (30)
cis-Stilbene	Naringenin	Allyl-thiourea
trans-Stilbene	Naringin	Amiodarone
Azulene	Progesterone	5α-Androstane
Dibenzyl	Resveratrol	Benzophenone
Acridine	Retene	Benzoyl peroxide (BPO)
Flavone	9-cis-Retinoic Acid	Camphorquinone
Biochanin A	13-cis-Retinoic acid	Cinnarizine
Quercetin Dihydrate	all-trans-Retinal	Clofibrate
Genistein	all-trans-Retinoic acid	Clomiphene
17β-Estradiol	all-trans-Retinol	Cyproterone acetate
Zearalenone	3,5-Diiodo-L(+)-tyrosine dihydrate	Dexamethasone
Daidzein	T3 (3,5,3'-Triiodothyronine)	Dienestrol
β-Estradiol 3-Sulfate	T4 (3,3',5,5'-Tetraiodothyronine)	Diethylstilbestrol (DES)
β-Estradiol 3,17-Disulfate	3,3',5'-Triiodo-L-Thyronine	Dimethylaminoethyl methacrylic acid
β-Estradiol 17-(β-D-Glucuronide)	Vitamin A acetate	Dimetyl-p-toluidine
β-Estradiol 3-(β-D-Glucuronide)		Ethynylestradiol
Testosterone	Natural product-related chemicals (37)	Flutamide
Kaempferol	6,8-Dichlorochrysin	Hydroxypropionic Acid
Abietic Acid	6,8-Dichloroapigenin	4-Hydroxytamoxifen
Phloretin	3',8-Dichlorodaidzein	ICI 182780
Apigenin	3',5',8-Trichlorodaidzein	3-Iodo-L-tyrosine
4',5,7-Trihydroxyflavanone	6,8-Dichlorogenistein	Methyltrienolone
Coumestrol	6,8-Dichloronaringenin	Mibolerone
Genistin	6,8-Dichlorocatechin	5-Propyl-2-thiouracil
Daidzin	2-Chloroestrone (E1)	6-n-Propyl-2-thiouracil
17α-Estradiol	4-Chloroestrone (E1)	Spironolactone
Chrysin	2,4-Dichloroestrone (E1)	Tamoxifen
Luteolin	2,4,16,16-Tetrachloroestrone (E1)	3,3',5,5'-Tetraiodothyroacetic acid
Indole-3-Carbinol	10-Chloro-1,4-estradiene-3,17-dione	Thiamazole
Hesperetin	2-Chloro-17β-estradiol (E2)	3,3',5-Triiodothyroacetic acid
β-Sitosterol	4-Chloro-17β-estradiol (E2)	
Equol	2,4-Dichloro-17β-estradiol (E2)	Cosmetic-related chemicals (28)
Enterolactone	2-Chloroestriol (E3)	4-Aminobenzoic Acid
Formononetin	4-Chloroestriol (E3)	2-phenoxyethanol
β-Ecdysterone	2,4-Dichloroestriol (E3)	Ethyl 4-aminobenzoate
α-Ecdysterone	2-Chloro-17α-ethinylestradiol (EE2)	2-Ethylhexyl-4-p-dimethylamino-benzoate
Juvabione	4-Chloro-17α-ethinylestradiol (EE2)	2-Hydroxyethyl salicylate
Cyasterone	2,4-Dichloto-17α-ethinylestradiol (EE2)	4-t-Butylphenyl salicylate
Murisuterone A	4-Androstene-3,17-dione	4-Octylphenyl salicylate
Allylisothiocyanate	16,16-Dichloro-4-androstene-3,17-dione	Salicylic Acid 2-Ethylhexyl Ester
Catechin	1,4-Androstadiene-3,17-dione	2,4-Dihydroxy-benzophenone
Dehydroabietic acid	4,6-Cholestadien-3-one	2-Hydroxy-4-methoxy-benzophenone
5α-Dihydrotestosterone	2-Bromoestrone	2,2'-Dihydroxy-4-methoxy-benzophenone
Estriol	4-Bromoestrone	2,2',4,4'-Tetrahydroxybenzophenone
Estrone	2,4-Dibromoestrone	2,2'-Dihydroxy-4,4'-dimethoxy-benzophenone
Fisetin hydrate	2-Bromo-17β-estradiol	2-Hydroxy-4-n-octyloxy-benzophenone
Flavanone	4-Bromo-17β-estradiol	2-Hydroxy-4-methoxy-benzophenone-5-sulfonic acid
Galangin	2,4-Dibromo-17β-estradiol	4-t-Butyl-4'-methoxy-dibenzoylmethane
Harmol hydrochloride dihydrate	2-Bromoestriol	2-(2'-Hydroxy-5'-methylphenyl)-benzotriazole
Hesperidin	4-Bromoestriol	2-(2'-Hydroxy-5'-tert-butylphenyl)-benzotriazole
Hinokitiol	2,4-Dibromoestriol	2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)-benzotriazole
Hydrocortisone	2-Bromo-17α-ethinylestradiol	2-(2'-Hydroxy-3',5'-di-tert-amylphenyl)-benzotriazole
6-Hydroxyflavanone	4-Bromo-17α-ethinylestradiol	2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole
7-Hydroxyflavanone	2,4-Dibromo-17α-ethinylestradiol	2-(5-Chloro-2-benzotriazolyl)-6-tert-butyl-p-cresol
16α-Hydroxyestrone		3-(4-Methylbenzylidane)-camphor
11-Ketotestosterone		2-Ethylhexyl-4-Methoxycinnamate
		(±)-α-Tocopheryl Acetate
		Octamethylcyclotetrasiloxane
		Decamethylcyclopentasiloxane
		Dodecamethylcyclohexasiloxane

of RAR, was used as a standard. A DMSO solution of *all-trans* RA was stored in a shielding container at −80 °C until just before examination to prevent degradation, and seven serial twofold concentrations of *all-trans* RA were examined for every culture of yeast cells. A dose-response curve of the luminescence intensity of each compound was described, and two activity values were calculated from the power approximate expression. The EC × 10 was defined as the concentration of a test solution producing luminescence intensity 10 times that of the blank control, and the REC20 (20% relative effective concentration) was the concentration showing 20% of the activity of 10^{-8} M *all-trans* RA. Activity relative to RA was then calculated by dividing the REC20 of *all-trans* RA by that of a test compound. As there were no high volatile compounds in this study, loss of compounds by volatilization over an incubation period was not taken into consideration.

3. Results

3.1. Assay characteristics

The sensitivity and reproducibility of the RAR yeast assay were assessed using the endogenous ligand, *all-trans*

RA. As a chemiluminescence method is used in which an artificial substrate for β-galactosidase is added to sensitively detect transcriptional activation via the human RAR, the large amount of substrate did not allow luminescence intensity to reach a plateau, even at high concentrations of *all-trans* RA (Fig. 1). Therefore, the half-maximal effective concentration (EC_{50}) commonly used in this kind of *in vitro* assay was not reasonable to evaluate the agonistic ability of test chemicals in the present method. Instead of the EC_{50}, the EC × 10 of *all-trans* RA as defined above was 5.41 ± 1.73 nM (mean ± SD, 22 experiments, Table 2). Chemiluminescence intensity at 10^{-8} M *all-trans* RA was 21.2 ± 7.2 times that of the blank control, and then the REC20 was 2.19 ± 0.20 nM (Table 2).

3.2. Positive substances

Eighty-five of the 543 tested compounds, at their highest concentrations (10 μM), exhibited transcriptional agonistic activity via the human RARγ of at least 20% that of 10^{-8} M *all-trans* RA. Table 2 lists the positive substances in order of RARγ activation potency grouped into categories as for Table 1. The range of molecular weights was 164 (4-*n*-pentylphenol) to 444 (*trans*-nonachlor), and the range of total carbon number was 7 (chlorpyrifosmethyl) to 20 (*all-trans* retinol). Many of the tested organochlorine pesticides, styrene dimers, monoalkylphenols and parabens were found to be RARγ active.

3.3. Organochlorine pesticides

Sixteen of the tested 30 organochlorines had a positive effect on RARγ transfected yeast cells. γ-BHC was the most potent in this category and the activities of this compound and seven other organochlorines were over 0.1% (1/1000) of that of *all-trans* RA. The numbers of chlorine atoms in the active compounds were 6–9, while organochlorines out of this range had no effect on the RARγ.

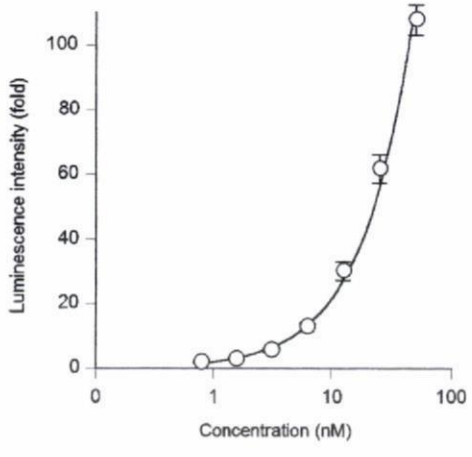

Fig. 1. Response of a yeast two-hybrid assay transfected with the human RARγ and the coactivator to the endogenous ligand, *all-trans* retinoic acid. Values are presented as n-fold induction over the vehicle control and as the mean ± SE of eight independent duplicate experiments.

3.4. Styrene dimers and trimers

All tested styrene dimers and a styrene dimer mixture of unspecified composition exhibited agonistic activity on the RARγ (Fig. 2), but styrene trimers had no effect. 1-Phenyltetralin and 1-methyl-3-phenylindan were particularly active styrene dimers with activities of over 0.6% of that of *all-trans* RA, while five other compounds had agonistic activities over 0.1%.

3.5. Monoalkylphenols

There were several highly active monoalkylphenols with 4-*n*-heptylphenol being the most potent compound tested in this study. All active monoalkylphenols had their alkyl chains *para* to the phenolic hydroxyl group, and the five most active compounds in this category had an alkyl group containing 6–9 carbons and their activities were over 0.4% of that of *all-trans* RA. The ranking of phenols having a linear alkyl group was heptyl (7 carbons) > hexyl (6) > octyl (8) > pentyl (5) > nonyl (9) > dodecyl (12) (Fig. 3). Branching of the alkyl group altered the potency of the phenols (Fig. 4). Comparison of REC20 values showed that an unspecified mixture of isomers of 4-nonylphenol was 4.7 times as active as 4-*n*-nonylphenol and that 4-tert-octylphenol was 2.2 times as active as 4-*n*-octylphenol. However, 4-tert-pentylphenol was 2.8-fold weaker than 4-*n*-pentylphenol. Moreover, six ring-chlorinated monoalkylphenols exhibited positive effects and had about the same or more potency than their unchlorinated analogs.

3.6. Parabens and other chemicals

Some alkyl *p*-hydroxybenzoates (parabens) with an alkyl group of 4–8 carbons were also positive to the RARγ. Of six positives, *n*-hexyl 4-hydroxybenzoate, *n*-pentyl 4-hydroxybenzoate and benzyl 4-hydroxybenzoate showed the highest agonistic activities with values over 0.1% that of *all-trans* RA. Phenols having a cyclic hydrocarbon side-chain *para* to the phenolic hydroxyl group (except 2-(1-adamantyl)-4-methylphenol), a hydroxyethyl ether of 4-nonylphenol and its chlorinated derivative were also positive to the RARγ, but with the exception of 4-(1-adamantyl)phenol, their activities were under 0.1% of that of *all-trans* RA. There were six active substances in the group that we have categorized as 'bisphenol-related compounds', namely, chlorinated bisphenol A and compounds found as impurities in industrial grade bisphenol A, but their activities, with the exception of 4-cumylphenol, were comparatively low. As shown in Table 2, there were also several active substances in the other categories, but most of these had a low potency for the RARγ. However, three diphenyl ethers, aclonifen, nitrofen and chlornitrofen, and the

Table 2
Responsiveness of a RARγ yeast assay to active compounds

Compounds	CAS No.	EC×10 (± SD, ×10^{-6} M)	REC20 (± SD, ×10^{-6} M)	Activity relative to RA (%)
all trans-Retinoic acid	302-79-4	0.00541 ± 0.00173	0.00219 ± 0.00020	100
Industrial chemicals (55)				
Aromatic hydrocarbons (4)				
2-Terphenyl	84-15-1	5.03 ± 2.43	1.77 ± 1.14	0.165
n-Octylbenzene	2189-60-8	20.09 ± 6.88	4.51 ± 2.52	0.065
1,3-Diphenylpropane	1081-75-0	8.68 ± 0.81	4.74 ± 1.10	0.062
Triphenylmethane	519-73-3	19.73 ± 4.76	5.34 ± 1.10	0.055
Bisphenols and related chemicals (6)				
2,2'-Dichlorobisphenol A	-	18.95 ± 7.05	6.15 ± 1.61	0.048
2,2',6-Trichlorobisphenol A	-	16.66 ± 5.30	7.85 ± 1.56	0.037
4-Cumylphenol	599-64-4	2.73 ± 0.58	1.68 ± 0.67	0.174
2-(4-Hydroxyphenyl)-2,4,4-trimethylchroman	-	6.53 ± 0.44	3.97 ± 1.33	0.074
2-(2-Hydroxyphenyl)-2,4,4-trimethylchroman	-	7.26 ± 0.96	4.57 ± 2.15	0.064
4-(4-Hydroxyphenyl)-2,2,4-trimethylchroman	472-41-3	6.98 ± 1.33	5.11 ± 2.00	0.057
Monoalkyl phenols and related chemicals (15)				
4-n-Heptylphenol	1987-50-4	0.49 ± 0.26	0.21 ± 0.11	1.363
4-t-Octylphenol	140-66-9	0.78 ± 0.41	0.29 ± 0.13	0.997
4-n-Hexylphenol	2446-69-7	0.69 ± 0.23	0.42 ± 0.13	0.695
4-Nonylphenol (mixed isomers)	84852-15-3	1.36 ± 0.70	0.62 ± 0.25	0.476
4-n-Octylphenol	1806-26-4	1.70 ± 0.56	0.66 ± 0.41	0.446
4-n-Pentylphenol	14938-35-3	3.43 ± 0.70	1.85 ± 0.44	0.159
4-n-Nonylphenol	104-40-5	4.61 ± 1.01	2.92 ± 1.10	0.100
4-Dodecylphenol (mixed isomers)	27193-86-8	5.45 ± 0.84	3.62 ± 0.72	0.081
4-t-Pentylphenol	80-46-6	9.92 ± 2.29	5.24 ± 1.33	0.056
2-Chloro-4-octylphenol	-	0.61 ± 0.37	0.23 ± 0.05	1.286
2,6-Dichloro-4-octylphenol	-	0.70 ± 0.16	0.28 ± 0.14	1.041
2-Chloro-4-nonylphenol	-	1.70 ± 0.96	0.69 ± 0.06	0.422
2,6-Dichloro-4-nonylphenol	-	2.77 ± 1.25	1.35 ± 0.41	0.217
2,6-Dichloro-4-butylphenol	-	4.64 ± 0.32	3.05 ± 0.96	0.096
2-Chloro-4-butylphenol	-	17.33 ± 4.12	7.94 ± 1.04	0.037
Parabens (6)				
n-Hexyl 4-hydroxybenzoate	1083-27-8	1.24 ± 0.26	0.75 ± 0.26	0.391
n-Amyl 4-hydroxybenzoate	6521-29-5	2.95 ± 0.26	1.92 ± 0.58	0.153
Benzyl 4-hydroxybenzoate	94-18-8	3.18 ± 0.55	2.26 ± 0.64	0.130
Isobutyl-4-hydroxybenzoate	4247-02-3	5.54 ± 0.93	4.08 ± 1.36	0.072
2-Ethylhexyl 4-Hydroxybenzoate	5153-25-3	5.58 ± 0.76	4.27 ± 0.90	0.069
Butyl-4-hydroxybenzoate	94-26-8	10.35 ± 2.66	6.37 ± 1.80	0.046
Phenols and related chemicals (7)				
4-(1-Adamantyl)phenol	29799-07-3	1.30 ± 0.80	0.54 ± 0.33	0.547
2-Chloro-4-nonylphenyl 2-hydroxyethyl ether	-	4.93 ± 1.33	3.21 ± 0.31	0.091
4-Cyclohexylphenol	1131-60-8	5.48 ± 1.00	3.23 ± 1.06	0.091
2-(1-Adamantyl)-4-methylphenol	41031-50-9	4.90 ± 0.68	3.82 ± 0.55	0.077
4-Nonylphenyl 2-hydroxyethyl ether	-	7.61 ± 2.34	4.35 ± 0.76	0.067
4-Benzylphenol	101-53-1	9.39 ± 3.55	4.74 ± 1.07	0.062
Hexestrol	5635-50-7	22.01 ± 3.51	7.00 ± 2.18	0.042

aromatic hydrocarbon 2-terphenyl showed agonistic activities over 0.1% of that of *all-trans* RA.

4. Discussion

The reactivity, reproducibility and dose-dependency of the RARγ yeast assay, using *all-trans* RA, as a standard, were satisfactory for assessing a wide range of chemicals as described here. The EC_{50} of *all-trans* RA in a reporter gene assay using RARγ-cotransfected HeLa cells and the IC_{50} in a competitive binding assay using RARγ-transfected COS-1 cells were reported to be 2.5 nM and 8 ± 1 nM, respectively (Bernard et al., 1992; Allenby et al., 1994). Although there is no simple comparison between these reports and our results, the responsivity of our assay system, which can be represented as EC × 10 or REC20 values, is within a similar range. Taking into account its simplicity and rapidity, application of this assay using easily managed yeast cells to the toxicological evaluation of chemicals is appropriate and reasonable as a step

Table 2 (*continued*)

Compounds	CAS No.	EC×10 (± SD, $\times 10^{-6}$ M)	REC20 (± SD, $\times 10^{-6}$ M)	Activity relative to RA (%)
all trans-Retinoic acid	302-79-4	0.00541 ± 0.00173	0.00219 ± 0.00020	100
Industrial chemicals (continued)				
Styrene dimers (14+1)				
1-Phenyltetralin	-	0.65 ± 0.02	0.25 ± 0.16	1.169
1-Methyl-3-phenylindan	-	1.22 ± 0.13	0.46 ± 0.28	0.632
trans-1,2-Diphenylcyclobutane	20071-09-4	3.37 ± 0.80	1.33 ± 0.75	0.220
1-Methyl-1-phenylindan	79034-12-1	4.77 ± 1.98	2.16 ± 0.81	0.135
cis-1,2-Diphenylcyclobutane	7694-30-6	5.43 ± 2.03	2.20 ± 1.12	0.133
2,4-Diphenyl-1-butene	16606-47-6	4.62 ± 0.69	2.32 ± 0.52	0.126
2,3-Diphenyl-1-butene	-	5.51 ± 2.81	2.40 ± 1.35	0.122
cis-1,4-Diphenyl-1-butene	-	6.22 ± 2.78	2.95 ± 1.38	0.099
trans-1,3-Diphenyl-1-butene	-	6.04 ± 1.91	2.99 ± 1.39	0.098
trans-1,3-Diphenylcyclobutane	-	7.09 ± 2.15	3.23 ± 1.39	0.091
1,4-Diphenyl-2-butene	-	8.29 ± 4.00	3.54 ± 1.62	0.083
cis-2,4-Diphenyl-2-butene	-	8.95 ± 2.77	3.89 ± 1.76	0.075
trans-1,4-Diphenyl-1-butene	-	8.62 ± 3.77	4.09 ± 1.84	0.072
trans-2,4-Diphenyl-2-butene	-	14.33 ± 4.49	6.83 ± 2.40	0.043
Polystyrene standard (mixed styrene dimer isomers)	-	7.54 ± 2.96	3.97 ± 0.77	0.074
Others (2)				
Octachlorostyrene	29082-74-4	21.93 ± 9.01	4.77 ± 3.47	0.061
Benzyl-2-naphthylether	613-62-7	21.98 ± 6.32	6.79 ± 0.37	0.043
Agrochemicals (22)				
Carbamate (1)				
Thiobencarb	28249-77-6	16.74 ± 1.64	5.48 ± 0.39	0.054
Diphenyl ethers (3)				
Aclonifen	74070-46-5	2.74 ± 0.62	1.33 ± 0.82	0.220
Nitrofen	1836-75-5	4.45 ± 1.31	2.18 ± 0.61	0.134
Chlornitrofen (CNP)	1836-77-7	7.77 ± 3.31	2.82 ± 1.22	0.104
Organochlorines (16)				
1,2,3,4,5,6-Hexachlorocyclohexane (γ-BHC)	58-89-9	0.89 ± 0.27	0.44 ± 0.14	0.668
Endrin	72-20-8	1.22 ± 0.79	0.85 ± 0.21	0.346
Heptachlor	76-44-8	2.09 ± 0.77	0.87 ± 0.37	0.336
Oxychlordane	27304-13-8	3.87 ± 0.99	1.90 ± 0.03	0.154
Chlordane	57-74-9	3.23 ± 1.89	1.93 ± 1.15	0.152
Toxaphene	8001-35-2	3.35 ± 1.64	2.59 ± 0.96	0.113
cis-Heptachlor epoxide	1024-57-3	3.46 ± 1.82	2.85 ± 0.60	0.103
cis-Chlordane	5103-71-9	5.63 ± 4.03	2.94 ± 1.33	0.100
trans-Chlordane	5103-74-2	7.15 ± 3.70	3.56 ± 1.80	0.082
β-Endosulfan	33213-65-9	9.06 ± 0.87	4.36 ± 2.71	0.067
Dieldrin	60-57-1	7.47 ± 4.60	4.70 ± 1.80	0.062
trans-Nonachlor	39765-80-5	7.74 ± 5.15	4.92 ± 0.30	0.060
Aldrin	309-00-2	8.88 ± 6.14	5.43 ± 0.91	0.054
β-Benzene hexachloride (β-BHC)	319-85-7	12.85 ± 3.15	5.69 ± 0.77	0.052
α-Endosulfan	959-98-8	8.02 ± 2.67	6.07 ± 2.40	0.048
trans-Heptachlor epoxide	1024-57-3 (trans)	8.49 ± 5.06	6.30 ± 1.58	0.047
Organophosphates (2)				
Cyanofenphos	13067-93-1	8.83 ± 1.51	3.42 ± 1.89	0.086
Chlorpyrifosmethyl	5598-13-0	13.81 ± 3.47	7.08 ± 2.11	0.041
Natural compounds and related chemicals (7)				
all-trans-Retinol	68-26-8	2.81 ± 0.17	1.67 ± 0.77	0.175
Flavanone	487-26-3	10.65 ± 1.14	4.23 ± 0.26	0.069
cis-Stilbene	645-49-8	10.16 ± 5.57	4.99 ± 2.58	0.059
Dibenzyl	103-29-7	17.95 ± 3.31	6.66 ± 2.10	0.044
4-Chloro-17β-estradiol	-	16.23 ± 3.77	4.35 ± 1.75	0.067
2,4-Dichloro-17β-estradiol	-	13.26 ± 1.52	4.79 ± 1.38	0.061
4-Bromo-17β-estradiol	-	12.49 ± 4.72	5.52 ± 3.91	0.053
Medicines and cosmetic-related chemicals (1)				
Cosmetic-related chemical (1)				
3-(4-Methylbenzylidane)-camphor	36861-47-9	15.98 ± 4.04	5.92 ± 1.85	0.050