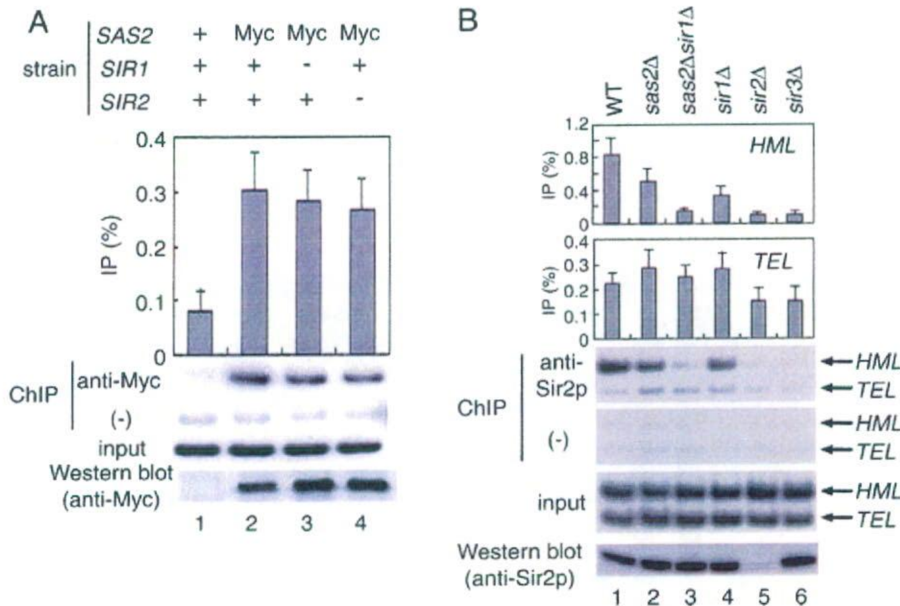

Figure 4. (**A**) *SIR1* and *SIR2* are not required for the recruitment of Sas2p to the promoter in the *HMLα* locus. ChIP assay was performed with chromatin prepared from *sir1Δ* (YSM85) and *sir2Δ* (YSM90) strains that expressed the C-terminal Myc epitope-tagged Sas2p. (**B**) Effect of deletion of *SAS2* and *SIR* genes on the Sir2p occupancy. Soluble chromatin was prepared from wild-type and deletion strains, and immunoprecipitated with or without anti-Sir2p antibody. Strains analyzed (ordered from left to right) were W303-1a, *sas2Δ* (YJW253), *sas2Δsir1Δ* (YJW258), *sir1Δ* (YJW252), *sir2Δ* (YSM64) and *sir3Δ* (YS480). Results are shown as in Figure 3B.

deletion strains (Figure 4A). We prepared chromatin fractions from these strains and subjected them to ChIP analysis for the Myc epitope. As shown previously, Sas2p–Myc associated with the *HMLα* region in wild-type cells. Loss of *SIR1* or *SIR2* did not affect this association (Figure 4A). This finding indicates that *SIR1* and *SIR2* are not required for the recruitment of Sas2p to the promoters in the *HMLα* locus.

Disruption of *SAS2* in a wild-type strain increased the spreading of Sir proteins to the sub-telomeric region (15,16). Deletion of *SAS2* in a *sir1* mutant also may lead to spreading of the finite number of Sir2p molecules into sub-telomeric regions, resulting in a decrease in Sir2p occupancy at the *HMLα* locus. To test this hypothesis, we asked whether loss of *SAS2* results in increased or decreased Sir2p association. We observed localization of Sir2p in the regions of the *HMLα* locus and sub-telomeric chromatin 7.5 kb from the end of chromosome VI in the wild-type and deletion strains (Figure 4B). Consistent with previous observations, the Sir2p association detected within the *HMLα* locus and sub-telomeric regions in the wild-type strain was greater than that in a *sir2* deletion strain (Figure 4B, compare lanes 1 and 5) and was slightly increased in the sub-telomeric region by *SAS2* deletion (16,29). Interestingly, although Sir2p was expressed efficiently in all of the strains except a *sir2* deletion strain, the disruption of *SAS2* or *SIR1* decreased the amount of Sir2p localization at the *HMLα* locus (Figure 4B). In the combination of *SAS2* deletion with the null allele of *SIR1*, the Sir2p association at the *HMLα* locus was completely lost, similar to that in the *SIR2* and *SIR3* deletion strains. This indicates that although Sas2p association at the *HMLα* locus does not require *SIR1* or *SIR2*, Sir2p localization is partially dependent on *SAS2*.

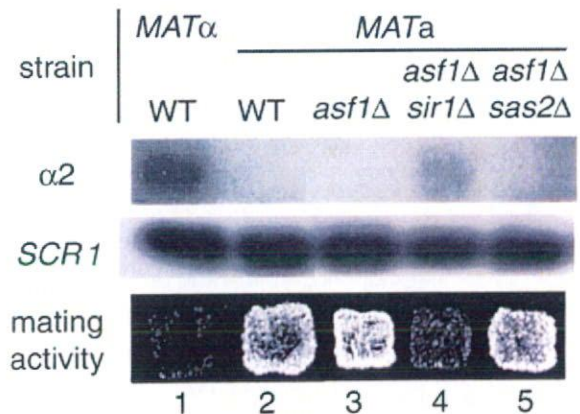

Figure 5. *ASF1* and *SAS2* function in the same pathway in *HMLα* silencing. Disruption of *ASF1* in combination with a null allele of *SIR1*, but not of *SAS2*, derepressed α2 expression. RNA from either wild-type (WT: W303-1b and W303-1a), *asf1Δ* (YJW433), *asf1Δsir1Δ* (YJW435) or *asf1Δsas2Δ* (YJW436) was hybridized by northern blotting to a probe specific for either the α2 or *SCR1* gene. A qualitative mating assay was performed by patches, which were replica-plated to a lawn of α cells.

α2 expression in the *asf1 sir1* deletion strain

We and others (11,12) previously showed that the SAS complex physically interacts with Asf1p and these factors function in a pathway that enhances the epigenetic silencing defects of *sir1* mutants. To learn more about the function of *ASF1* in the *HMLα* silencing, we measured the expression of α2 in an *ASF1* deletion strain (Figure 5). Deletion of *ASF1* results in a very slight reduction in silencing at *HMLα* as indicated by

quantitative mating analysis (11), and the α2 mRNA level in the *asf1* mutant was indistinguishable from that of the wild-type strain. We previously showed that the combined deletion of *ASF1* and *SIR1* caused much more severe silencing defects at *HMLα* than does the deletion of *ASF1* alone (11). Derepression of α2 repression occurred in the *asf1 sir1* double-deletion strain but not in the *asf1Δsas2Δ* strain. Loss of mating activity correlated with the increased α2 expression in the *asf1 sir1* deletion strain (Figure 5).

Loss of *ASF1* disrupts the recruitment of the SAS complex to the *HMLα* locus

We showed that *SIR1* and *SIR2* are not required for the recruitment of Sas2p to the promoters in the *HMLα* locus (Figure 4). Next, we investigated whether *ASF1* is required for Sas2p–Myc recruitment. Loss of *ASF1* markedly decreased Sas2p association with the *HMLα* locus (Figure 6A). This decrease was restored by a plasmid carrying *ASF1* (compare lanes 3 and 4). In a *sir1* mutant, the effect of *ASF1* on the Sas2p recruitment was the same as for the *SIR1* wild-type strain. These results indicate that *ASF1*, but not *SIR1*, is required for the recruitment of Sas2p to the *HMLα* locus. Sas2p expression levels in whole-cell extracts from wild-type, *asf1Δ*, and *asf1Δsir1Δ* strains were indistinguishable, and disruption of *ASF1* did not affect the size of the SAS complex (Figure 6A and B). These data indicate that loss of the association of Sas2p with the *HMLα* locus in the *asf1* mutants is not due to a decrease in Sas2p expression or disruption of the SAS complex.

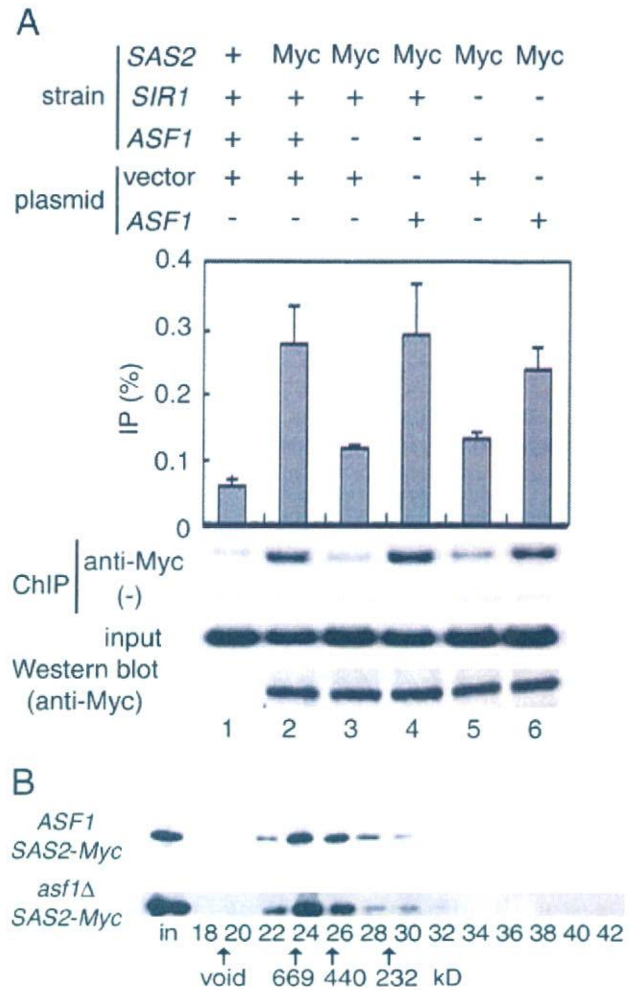

Figure 6. Sas2 occupancy at the *HMLα* region is dependent on *ASF1*. (**A**) Chromatin was prepared from the Sas2p–Myc-expressing strains containing either an empty vector or a CEN-based *ASF1* plasmid expressed from its own promoter. Strains for the ChIP assay (ordered from left to right) were YSM112, YSM113, YSM114, YSM115, YSM116 and YSM117. Results are shown as in Figure 3B. (**B**) Comparison of the Sas2p–Myc elution profiles from Superdex 200 size exclusion chromatography after fractionation of whole-cell extracts prepared from wild-type (YJW265) and *asf1Δ* (YSM87) mutant strains. Shown are western blots of column fractions probed with the anti-Myc antibody.

DISCUSSION

Deletion of *SIR1* in combination with a null allele of either *SAS2* or *ASF1* causes a much more severe silencing defect at *HMLα* than does deletion of either gene alone (11), but the role of these factors in silencing was unclear. We showed that the combination of mutation of *SAS2* with *SIR1* induced derepression of α2 expression and changed the precisely positioned nucleosome that includes the transcriptional initiation site of the α2, and that the HAT activity of Sas2p is critical for this effect. Furthermore, ChIP assays revealed specific association of the SAS complex with the *HMLα* locus, and the SAS complex recruitment required *ASF1* but not *SIR1* and *SIR2*.

The effect of the disruption of *SAS2* on silencing is different among loci. For example, normal *HMR***a** silencing is unaffected by *SAS2* deletion, but *sas2* mutations suppress the silencing defect caused by mutation in the silencer elements of *HMR***a** (10,17). Deletion of *SAS2* leads to loss of hyperacetylation of histone H4 at lysine 16 in regions adjacent to telomeres. This results in the spreading of Sir3p away from the telomeres into these sub-telomeric regions, leading to repression of gene expression in the sub-telomeric region (15,16). However, deletion of *SAS2* causes the loss of silencing at the telomeres themselves, presumably because of titration of Sir proteins away from this locus.

In present study, Sas2p was found to be associated with the *HMLα* locus, and Sir2p was not required for this Sas2p association. Disruption of *SAS2* increased the spreading of Sir proteins to the sub-telomeric region (15,16). Deletion of *SAS2* in *sir1* mutants also led to the spreading of the finite number of Sir2p molecules into sub-telomeric regions and resulted in a decrease in Sir2p occupancy at the *HMLα* locus. We also showed that the Sas2p HAT activity is essential for α2 repression. Acetylation of lysine 16 of histone H4 might be a landmark for Sir2p assembly: once lysine 16 of histone H4 is acetylated by Sas2p, Sir2p recognizes and deacetylates that residue in the silenced domain and is held in this region. Sas2p might contribute to regulating the histone H4 lysine 16 acetylation state at the chromosome level as well as at the locus level. Deletion of *ASF1* likely would bring about the same phenomenon, because we found that Sas2p occupancy at the *HMLα* locus was dependent on *ASF1*. Other investigators have shown that the association of Sir proteins at the *HMLα* silencer is somewhat reduced in *sir1*-mutant cells (29). The deletion of

SIR1 in combination with null alleles of either *SAS2* or *ASF1* may decrease the association of Sir proteins to a much greater extent than that seen after deletion of *SIR1* only, thereby causing a much more severe silencing defect at *HML*α than that seen after deletion of either gene alone.

We previously showed that Sas4p, one of the subunits of the SAS complex, directly interacts with Asf1p (11). Therefore, recruitment of the SAS complex to the *HML*α region might require physical interaction with Asf1p. In the present study, we found that the SAS complex is associated with the *HML*α region, but not the *ACT1* promoter. However, Moshkin *et al.* (30) showed that *Drosophila* Asf1 associated with multiple sites, including heterochromatic and transcriptionally active regions. Furthermore, *asf1* mutants are defective in the repression of histone gene transcription during the cell cycle and in cells arrested in the early S phase (31). Finally, Asf1 interacts with bromodomain-containing subunits of TFIID and the Brahma complex, a member of the SWI/SNF ATP-utilizing chromatin-remodeling factors (30,32). These results indicate that *ASF1* affects transcriptional control through a variety of mechanisms. The estimated numbers of Asf1p and Sas4p molecules per yeast cell are 6230 and 768, respectively (33). This distribution suggests that the SAS complex interacts with a subset of Asf1 proteins. The mechanism of the recruitment specificity of the SAS complex is still unknown. *HML*α binding factors other than Sir proteins may enhance the SAS complex association with the *HML*α locus. Alternatively, Asf1p-associated factors that selectively bind to Asf1p within transcriptionally active regions may inhibit the interaction between the SAS complex and Asf1p. To address this possibility, we are purifying the factors that interact with the SAS complex and Asf1p.

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Conflict of interest statement. None declared.

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BIODEGRADATION OF BISPHENOL A AND DISAPPEARANCE OF ITS ESTROGENIC ACTIVITY BY THE GREEN ALGA *CHLORELLA FUSCA* VAR. *VACUOLATA*

TAKASHI HIROOKA,† HIROYASU NAGASE,*† KOTARO UCHIDA,† YUJI HIROSHIGE,† YOSHIE EHARA,† JUN-ICHI NISHIKAWA,‡ TSUTOMU NISHIHARA,‡ KAZUHISA MIYAMOTO,† and ZAZUMASA HIRATA,†

†Laboratory of Environmental Biotechnology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

‡Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

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Abstract—Bisphenol A (BPA) is known as an endocrine disruptor and often is found in landfill leachates. Removal of BPA by green alga, *Chlorella fusca*, was characterized, because we previously found that various phenols were well removed by this strain, including BPA. *Chlorella fusca* was able to remove almost all BPA in the concentration range from 10 to 80 μM for 168 h under continuous illumination at 18 W/m^2. At the low light intensity of 2 W/m^2, 82% of 40 μM BPA was removed, and only 27% was removed in the dark. Moreover, *C. fusca* could remove 90% of 40 μM BPA under the 8:16-h light:dark condition, which was almost as high as that under the continuous-light condition. The amount of BPA contained in the cells was less than the amount of BPA removed from the medium. Monohydroxybisphenol A was detected as an intermediate of BPA degradation. Moreover, estrogenic activity that originated from BPA in the culture medium also completely disappeared. Based on these results, BPA was finally degraded to compounds having nonestrogenic activity. Therefore, *C. fusca* can be considered a useful organism to remove BPA from landfill leachates.

Keywords—Bisphenol A *Chlorella fusca* Estrogenic activity Landfill leachate Monohydroxybisphenol A

INTRODUCTION

Microalgae frequently have been used in processes for removing pollutants, such as nitrogen and phosphorus, from wastewaters [1]. Biosorption of heavy metals and biodegradation of hazardous organic compounds by microalgae also have been reported [2,3]. In a previous study, we found that the green alga, *Chlorella fusca* var. *vacuolata* IAM C-28, which was obtained from the Institute of Applied Microbiology Culture Collection (Tokyo, Japan), could remove various kinds of phenols, such as chlorophenols, nitrophenols, and bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane), from medium [4].

Recently, BPA has often been detected in aquatic and soil environments [5,6]. Bisphenol A has an acute toxicity to aquatic organisms. It was reported that a median lethal concentration for fish is 3 to 15 mg/L and that a median effective concentration for green algae is 1 to 3 mg/L [6]. Because BPA also has endocrine-disrupting activity, it is hazardous to animals even at low concentrations [7,8]. Bisphenol A is widely used as a material to produce polycarbonate, epoxy resins, flame retardants, and other products. These plastics are finally disposed of into landfills, and BPA often is detected in landfill leachates at higher concentrations than those in environmental water [9,10]. Therefore, leachates are thought to be significant sources of BPA in aquatic environments. In Japan, landfill sites are categorized into three types depending on waste contents. Waste plastics, rubber, glasses, ceramics, nontoxic metals, and construction scrap materials are disposed of into landfill sites for stable industrial wastes. Industrial wastes containing toxic substances at high level are disposed of into strictly controlled landfills for industrial wastes. The other industrial and domestic wastes are disposed of into leachate-controlled landfills equipped with a leachate-treatment system. Urase et al. [11] and Yamada et al. [12] reported that more than 90% of the BPA contained in leachates from controlled-type landfill sites was removed by treatment processes consisting of activated sludge method, coagulation, sedimentation, sand filtration, and activated carbon adsorption. However, BPA also is detected in leachate from landfill sites for stable industrial wastes, which are not equipped with a leachate-treatment system [11,12]. Because the cost of the usual leachate-treatment system is high, a low-cost treatment system is required to remove BPA in leachates from open-landfill sites.

The present report focuses on the utilization of microalgae for removing BPA from landfill leachates. We evaluated the ability of *C. fusca* to remove BPA in batch experiments under light, dark, and light-dark cycle conditions. We also investigated the degradation of BPA by the cells, and then we identified an intermediate of BPA degradation. The disappearance of estrogenic activity originated from BPA in the culture medium also was analyzed by the yeast two-hybrid assay [13].

MATERIALS AND METHODS

Microorganism and culture condition

Chlorella fusca was precultivated in a modified Bristol medium at 27.5°C under continuous illumination with white fluorescent light at an intensity of 18 W/m^2 and aerated with air containing 1% CO_2. After 7 d, cells were harvested and inoculated into a 100-ml glass test tube as described previously [4]. Algal cells were cultivated under the same conditions as used for precultivation with 40 μM BPA as basal condition for BPA removal. To evaluate the ability of *C. fusca* for BPA

* To whom correspondence may be addressed
(nagase@phs.osaka-u.ac.jp).

removal, the BPA concentration was varied from 10 to 160 μM. To examine the effect of illuminating conditions on BPA removal, the light intensity was changed from 0 to 36 W/m^2 at the surface of the glass test tube, and the effect of a light-dark cycle (8:16-h light:dark photoperiod; light intensity, 18 W/m^2) also was investigated. The concentration of BPA and the cell density were measured every 24 h after addition of BPA. All the batch experiments were undertaken in triplicate.

Analytical methods

The cell growth was evaluated by the optical density at 680 nm and measured by a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). The concentration of BPA was measured using a high-performance liquid chromatography (HPLC) system (D-7000 series; Hitachi) with a diode-array detector (L-4500; Hitachi) at 280 nm. The pretreatment method for the HPLC sample was as follows: The culture (2 ml) was centrifuged (11,000 *g*, 4°C, 5 min), 200 μl of the supernatant were mixed with 20 μl of 6 N HCl to remove proteins, and a second centrifugation was performed (17,360 *g*, 4°C, 11 min). The supernatant (40 μl) was then injected into the HPLC system, which was equipped with a reversed-phase column (250 × 4.6 mm I. D., 5 mm; Mightysil RP-18; Kanto Chemical, Tokyo, Japan). Acetonitrile/50 mM potassium dihydrogenphosphate buffer (pH 2.5; 50/50 [v/v]) was used as the mobile phase at a flow rate of 0.7 ml/min. Degradation intermediates of BPA were identified using the HPLC–mass spectrometry (LC-MS) system (LCQ Advantage; Thermo Finnigan, San Jose, CA, USA) with an electrospray ionization interface. The HPLC sample (50 μl) was injected into the LC-MS system equipped with a reversed-phase column (250 × 4.6 mm I. D., 5 mm; L-column; Chemical Evaluation and Research Institute, Tokyo, Japan). Water/acetonitrile (60/40 [v/v]) was used as the mobile phase at a flow rate of 1.0 ml/min. The electrospray ionization interface was operated in a negative-ion mode.

Extraction of BPA from algal cells

Chlorella fusca was cultivated in the presence of 40 μM BPA in the light at 18 W/m^2. Algal cells were harvested from 5 ml of culture by centrifugation (845 *g*, 5°C, 5 min) and washed three times with distilled water. Cell pellets were re-suspended in 5 ml of distilled water. After ultrasonic disruption (Sonifier 450; Branson, Danbury, CT, USA) of the cells, 12.5 ml of methanol and 6.25 ml of chloroform were added. This solution was shaken for 1 h. Next, 6.25 ml of distilled water and 6.25 ml of chloroform were added to the solution, and the organic phase was collected by centrifugation (845 *g*, 5°C, 5 min). This treatment was repeated four times. Chloroform was evaporated completely from the organic phase by a rotary evaporator. The residual matter was dissolved in 10 ml of methanol and then analyzed by HPLC.

Chemical oxidation of BPA by Fremy's salt

Bisphenol A was oxidized by potassium nitrosodisulfonate (Fremy's salt; Sigma-Aldrich, St. Louis, MO, USA) to prepare monohydroxybisphenol A according to a previously reported method [14]. Fremy's salt (0.1 g) was added to 66 ml of 25 mM sodium dihydrogenphosphate buffer solution containing 13 mM BPA in a 200-ml Erlenmeyer flask. The mixture was shaken for 20 min at 25°C and then extracted with chloroform. After the organic phase was collected and chloroform evaporated, the residual matter containing red crystals was dissolved in 10 ml of ethanol. The solution was analyzed by LC-MS.

Estrogenic activity of the culture supernatant

The estrogenic activity of the culture supernatant was estimated by a two-hybrid yeast assay using the recombinant yeast *Saccharomyces cerevisiae* Y190 with pGBT9-ER LBD and pGAD424-TIF2 as described by Nishikawa et al. [13] with some modifications. Then, 250 μl of the supernatant of the *C. fusca* culture containing BPA or of that containing ethanol as a negative control were mixed with 200 μl of synthetic dextrose medium. The yeast, which had been precultured for 48 h, was added to the mixture at a volume of 50 μl and then cultivated for 4 h at 30°C in a culture-tube rotator (LD-76; Labinco B.V., Breda, The Netherlands) at 30 rpm. After the optical density at 595 nm (OD_{595}) of the culture was measured, the yeast cells were collected by centrifugation (1,100 *g*, 4°C, 5 min) from 350 μl of the culture and then resuspended in 200 μl of Z buffer (pH 7.0) containing 21.49 g of $Na_2HPO_4 \cdot 12H_2O$, 6.22 g of $NaH_2PO_4 \cdot 2H_2O$, 0.75 g of KCl, 0.246 g of $MgSO_4 \cdot 7H_2O$, 0.27 ml of β-mercaptoethanol, and 1 g of Zymolyase 20T (Seikagaku, Tokyo, Japan) in 1 L. Samples were incubated for 15 min at 37°C, and then enzymatic reaction was started by adding 40 μl of 4 g/L *o*-nitrophenyl-β-D-galactopyranoside. The mixtures were incubated for 90 min at 30°C, and 100 μl of 1 M Na_2CO_3 were added to stop the reaction. After centrifugation (17,360 *g*, 4°C, 5 min), the absorbance of the supernatant at 415 and 550 nm (A_{415} and A_{550}, respectively) was measured. Estrogenic activity was indicated as β-galactosidase activity (U) calculated by the following equation:

$$U = (A_{415} - (1.75 \cdot A_{550})) \times 10^3/(t \cdot v \cdot OD_{595})$$

where t is the time of reaction (90 min) and v is the volume of mixture used in the assay (0.05 ml).

RESULTS

Effects of initial BPA concentration on its removal and algal growth

The effect of initial BPA concentration on its removal was investigated. The concentration of BPA in uninoculated media was found not to decrease (data not shown). *Chlorella fusca* was cultivated with BPA in the concentration range from 10 to 160 μM for 168 h under the continuous-light condition (18 W/m^2). Figure 1 shows the BPA removal and cell growth. More than 95% of BPA was removed at the concentration between 10 and 80 μM, although only 70% of 160 μM BPA was removed. Therefore, it can be assumed that BPA was removed by the algal cells. This strain grew well in the BPA concentration range from 10 to 40 μM. Because the concentration of BPA in Japanese landfill leachates generally was lower than 40 μM [9–12], the initial BPA concentration was set at 40 μM for subsequent experiments.

Effects of light condition on BPA removal

To investigate the effects of light intensity on BPA removal, *C. fusca* was cultivated in the light-intensity range from 0 to 36 W/m^2 for 168 h (Fig. 2). The growth rates of this strain decreased with decreasing light intensity, and growth did not occur in the dark. The BPA removal was 98% at 36 W/m^2 and 82% at 2 W/m^2, although the removal decreased to 27% in the dark. Therefore, light illumination was an important parameter for achieving a high ability to remove BPA.

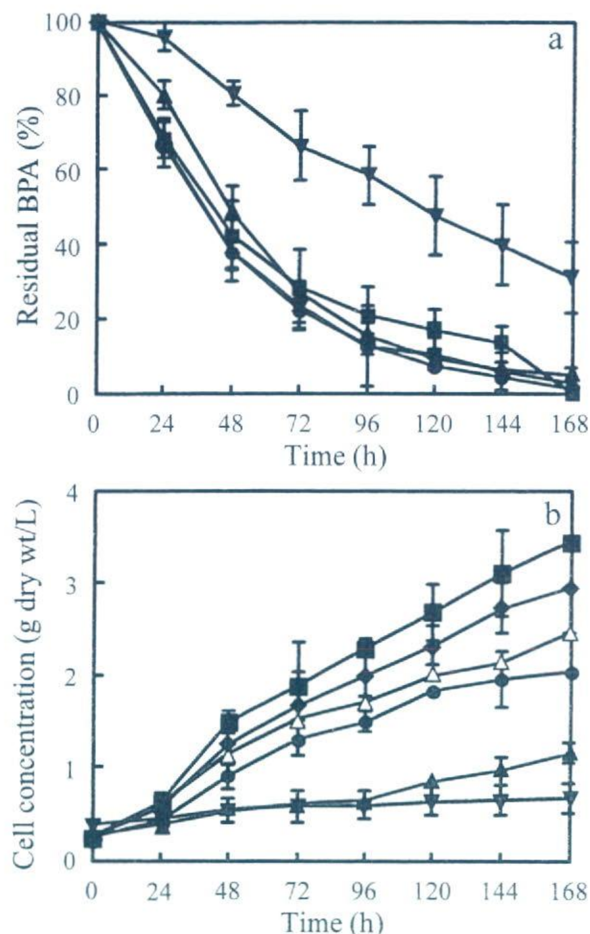

Fig. 1. Effect of initial bisphenol A (BPA) concentrations on its removal (**a**) and growth (**b**). The initial BPA concentrations were 0 μM (△), 10 μM (■), 20 μM (◆), 40 μM (●), 80 μM (▲), and 180 μM (▼). The light intensity was 18 W/m². Values were the averages of triplicate determinations. Error bars indicate standard deviations.

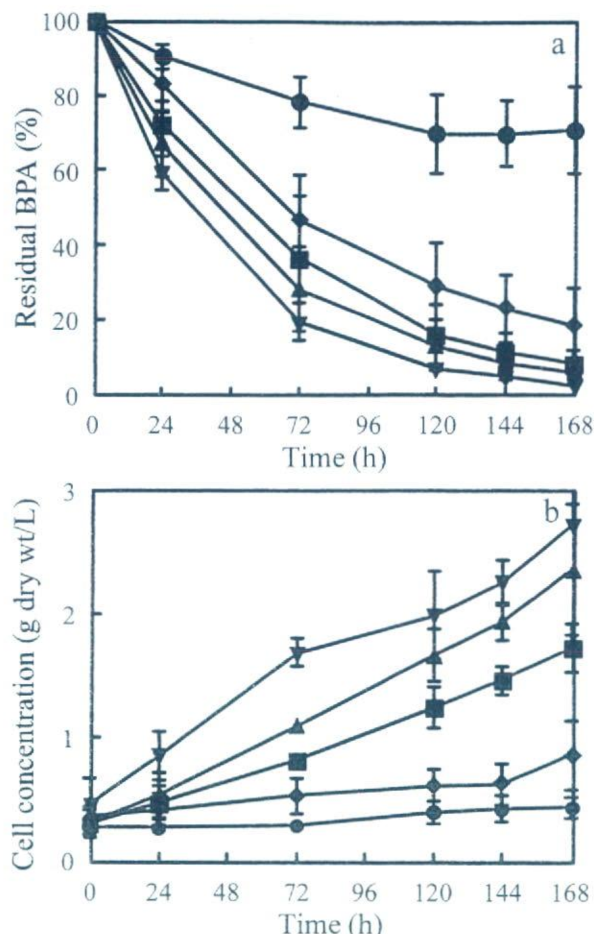

Fig. 2. Effect of light intensity on bisphenol A (BPA) removal (**a**) and growth (**b**). Light intensities tested were 0 W/m² (●), 2 W/m² (◆), 9 W/m² (■), 18 W/m² (▲), and 36 W/m² (▼). Values were the averages of triplicate determinations. Error bars indicate standard deviations.

Removal of BPA under the 8:16-h light:dark photoperiod (light intensity, 18 W/m²) also was investigated (Fig. 3). *Chlorella fusca* grew well and removed 90% of BPA, a result not very different from that obtained under continuous light (18 W/m²) for 168 h.

Biodegradation of BPA by C. fusca

To determine whether BPA was degraded by the cells or simply accumulated in the cells, the amount of BPA in the cells cultivated at 18 W/m² was analyzed (Fig. 4). The amount of BPA contained in the cells was significantly less than the amount of BPA removed from the medium. At the end of the cultivation time, BPA in the cells finally was decreased below the detection limit of HPLC analysis. This result indicates that BPA removal by *C. fusca* was caused by biodegradation by the cells rather than by simple accumulation in the cells.

On the HPLC chromatogram, an unknown peak at 8.2 min was observed in the culture medium in the light. A typical chromatogram is shown in Figure 5a. At 2 W/m², this peak area increased with decreasing BPA until 72 h and then decreased (data not shown). This peak was analyzed by LC-MS, and the mass spectrum of it revealed a parent-ion peak $[M-1]^-$ at m/z 243.11 (Fig. 6a). Because the molecular weight of BPA is 228.29, it was suggested that hydroxylation occurred in BPA. Based on these LC-MS results, this compound was thought to be monohydroxybisphenol A, an intermediate of BPA degradation by *C. fusca*. Atkinson and Roy [14] reported that monoquinone and monohydroxy derivatives of BPA were produced by BPA oxidation using Fremy's salt, strong oxidizing agent, and treatment with 1 N HCl. Therefore, monohydroxybisphenol A was produced according to their method and compared with the intermediate of BPA degraded by *C. fusca* using HPLC and LC-MS. The BPA degradation intermediate showed the same retention time (8.2 min) and mass spectrum as those of monohydroxybisphenol A (Figs. 5b and 6b).

Disappearance of estrogenic activity in the culture

To determine if estrogenic activity originated from BPA in the culture medium of *C. fusca* was decreased on BPA removal, the change of this activity was analyzed by yeast two-hybrid assay (Fig. 7). The activity decreased with decreasing BPA concentration and then completely disappeared.

DISCUSSION

In the present study, the green alga *C. fusca* well degraded BPA in the concentration range from 10 to 80 μM in the light (Fig. 1a). Yamamoto et al. [9] reported that the highest BPA concentration detected in landfill leachates was 75 μM, indicating that it is possible for *C. fusca* to remove BPA at this

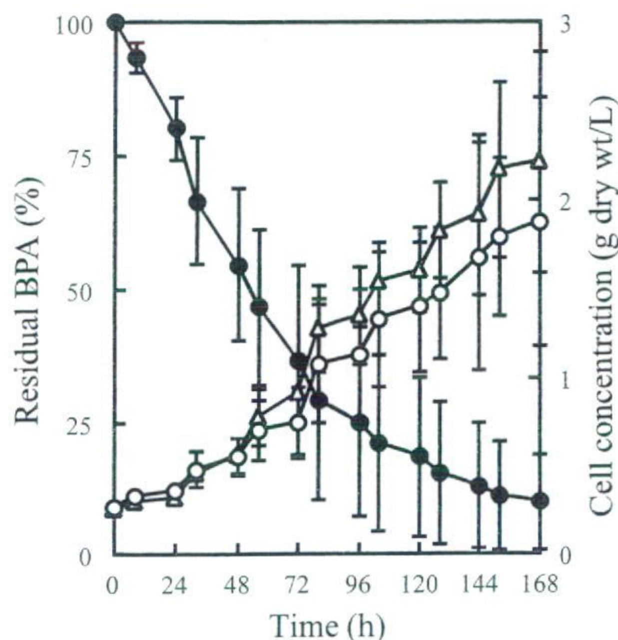

Fig. 3. Removal of bisphenol A (BPA) and growth under light-dark cycle condition. The BPA residual was shown (●). Algal cell concentration measured in media containing BPA (○) and not containing BPA (△), both incubated under 8:16-h light:dark photoperiod. Values were the averages of triplicate determinations. Error bars indicate standard deviations.

concentration. Several reports have appeared regarding the removal of BPA by bacteria. The gram-negative bacterium strain MV-1, isolated from the sludge of a wastewater treatment plant at a plastic manufacturing facility, was able to use BPA as a sole carbon and energy source. This bacterium removed 1.1 mM BPA completely from culture for 4 h [15]. Ronen and Abeliovich [16] reported that the gram-negative bacterium

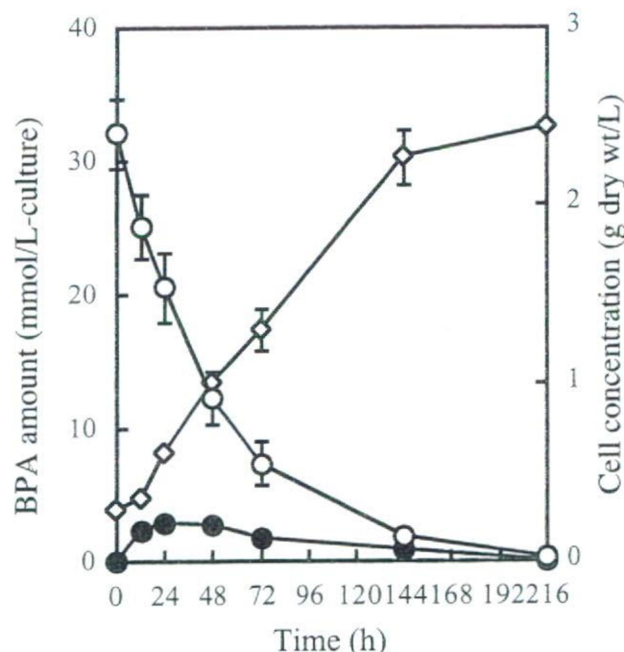

Fig. 4. Removal of bisphenol A (BPA) from the culture medium and change of its amount in the cells. Error bars indicate standard deviations. ○ = BPA amount in the culture medium; ● = BPA amount in cells; ◇ = cell amount.

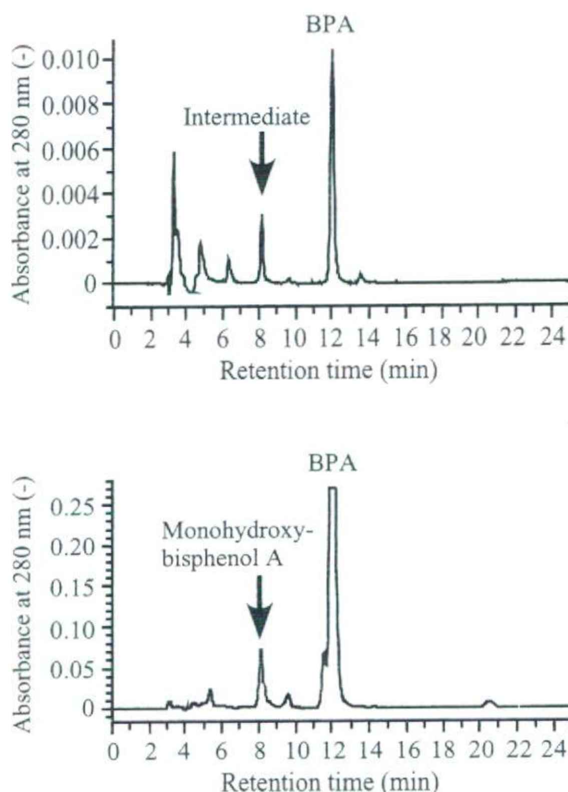

Fig. 5. High-performance liquid chromatograms of oxidized and degraded intermediate of bisphenol A (BPA) by *Chlorella fusca* (**top**) and Fremy's salt (**bottom**).

WH1, isolated from BPA-contaminated soil, removed 900 μM BPA completely from culture for 96 h. Kang and Kondo [17] reported that two *Pseudomonas* strains isolated from a river-water sample in Japan removed approximately 90% of 4 μM BPA for 240 h. However, these heterotrophic bacteria sometimes are difficult to use directly for treatment of BPA in landfill leachates, because the level of biologically available organic carbon sources usually is low. Therefore, it is necessary to add an external organic carbon source to maintain the biomass and the ability to remove BPA. On the other hand, microalgae can grow by using CO_2 as a carbon source. In this case, no need exists to supply any organic carbon sources. Therefore, *C. fusca* is thought to be useful to remove BPA contained in landfill leachates.

Chlorella fusca showed a high ability to remove BPA in the light-intensity range of 2 to 36 W/m^2, although this ability for removal decreased in the dark (Fig. 2). These results suggest that the light-illumination during cultivation is an important factor for the treatment of BPA by *C. fusca*. Bisphenol A also was removed in the light-dark cycle (Fig. 3), indicating that *C. fusca* would be a useful organism for BPA removal in the day-and-night cycle at most temperate or tropical outdoor sites.

As shown in Figure 4, the taken-up BPA was degraded in the cells. The degradation intermediate of BPA by *C. fusca* was identified as monohydroxybisphenol A, and this compound disappeared in the subsequent cultivation. Therefore, monohydroxybisphenol A would be a primary intermediate of BPA degradation in the light. In the case of bacteria, several intermediates of BPA degradation have been reported. The MV-1 strain has two pathways for BPA degradation [15,18]. The major pathway produces two primary intermediates, 4-

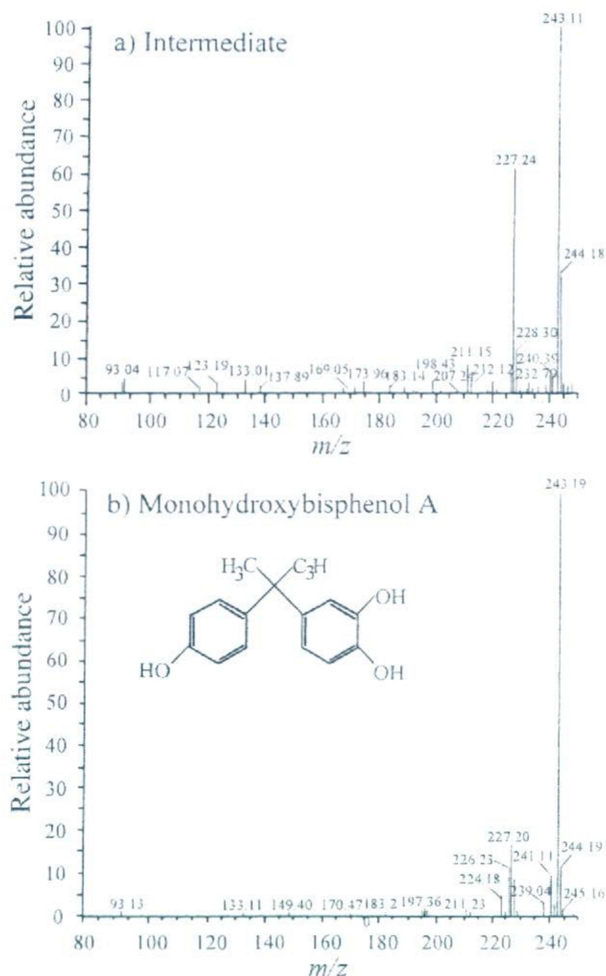

Fig. 6. Liquid chromatography–mass spectra of degraded intermediate of bisphenol A (BPA) by *Chlorella fusca* (**a**) and monohydroxybisphenol A (**b**).

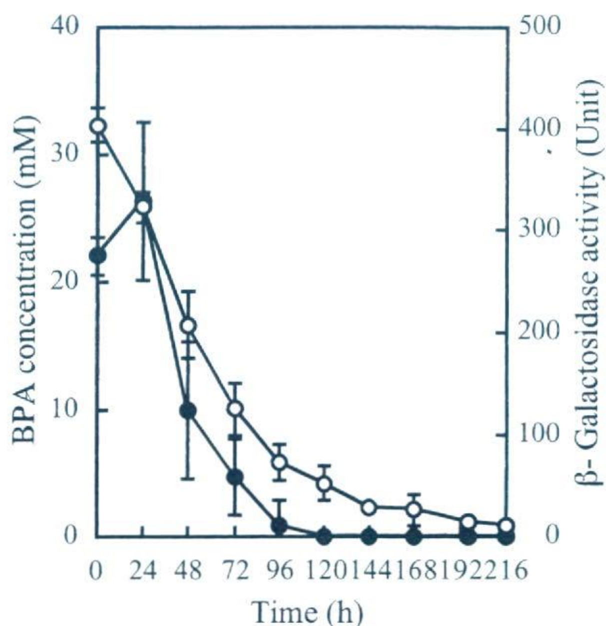

Fig. 7. Change of bisphenol A (BPA) concentration and estrogenic activity in the culture medium. Error bars indicate standard deviations. ○ = residual of BPA; ● = estrogenic activity in the culture medium.

hydroxybenzoic acid and 4-hydroxyacetophenone, and these intermediates were not detected in the case of *C. fusca*. On the other hand, monohydroxybisphenol A was reported as an intermediate of BPA degradation by potato (*Solanum tuberosum*) [19]. The BPA degradation pathway of *C. fusca* is similar to that in higher plants and not bacteria.

The reported degradation intermediates of BPA by heterotrophic bacteria have lower estrogenic activity compared with BPA [20]. The estrogenic activity also disappeared with the degradation of BPA by *C. fusca* (Fig. 7), indicating that *C. fusca* degrades BPA to intermediates with no estrogenic activity, including monohydroxybisphenol A. Therefore, it is possible to decrease the environmental effect of BPA using *C. fusca*.

The present study is, to our knowledge, the first to show that microalga *C. fusca* has the ability to degrade BPA. Microalgae have been employed to remove nitrogen and phosphorus in domestic wastewater by using a large-scale pond system. A similar system would be possible to remove BPA from landfill leachates, although further detailed studies will be required to achieve practical outdoor cultivation of *C. fusca* that maintain a high ability for BPA removal.

Acknowledgement—The present study was partly supported by a grant from the Heiwa Nakajima Foundation.

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Organotin Compounds Promote Adipocyte Differentiation as Agonists of the Peroxisome Proliferator-Activated Receptor γ/Retinoid X Receptor Pathway

Tomohiko Kanayama, Naoki Kobayashi, Satoru Mamiya, Tsuyoshi Nakanishi, and Jun-ichi Nishikawa

Departments of Environmental Biochemistry (T.K., N.K., S.M., J.N.) and Toxicology (T.N.), Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

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ABSTRACT

Nuclear receptors play important roles in the maintenance of the endocrine system, regulation of organ differentiation, and fetal development. Endocrine disruptors exert their adverse effects by disrupting the endocrine system via various mechanisms. To assess the effects of endocrine disruptors on nuclear receptors, we developed a high-throughput method for identifying activators of nuclear receptors. Using this system, we found that triphenyltin and tributyltin were activators of peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor. Because PPARγ is a master regulator of adipocyte differentiation, we assessed the effect of organotin compounds on preadipocyte 3T3-L1 cells. We found that organotin compounds stimulated differentiation of 3T3-L1 cells as well as expression of adipocyte marker genes.

An endocrine disruptor is an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or (sub)populations (WHO, 1996). Many naturally occurring and synthetic compounds, including DDT and its metabolites, polychlorinated biphenyls, and some alkylphenols, have hormonal activities (Sohoni and Sumpter, 1998; Nishihara et al., 2000; Gray et al., 2001; Sanderson et al., 2002). Although the levels of natural hormones are precisely regulated metabolically, synthetic chemicals elude this regulation to stimulate organs by mechanisms different from those of natural hormones.

The importance of nuclear receptors in endocrine function has been well established by many studies. The human genome contains at least 48 members of the nuclear receptor family (Chawla et al., 2001), and various chemicals bind to nuclear receptors and influence the expression of target genes (Blair et al., 2000; Sultan et al., 2001). To evaluate the effects of numerous synthetic chemicals on many nuclear receptors, we developed the CoA-BAP system, a high-throughput method for identifying nuclear receptor ligands (Kanayama et al., 2003). In the present study, we applied the CoA-BAP system to the evaluation of 16 human nuclear receptors and 40 suspected endocrine disruptors. We found that organotin compounds such as triphenyltin (TPT) and tributyltin (TBT) strongly activated retinoid X receptor (RXR) and PPARγ.

Organotin compounds have been used as agricultural fungicides, rodent repellents, and molluscicides and in antifouling paints for ships and fishing nets (Piver, 1973; Fent, 1996). These widespread uses have resulted in the release of increasing amounts of organotins into the environment. Although the toxicity of organotins has been reviewed extensively (Boyer, 1989), the molecular target of organotins has not yet been identified.

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ABBREVIATIONS: BAP, bacterial alkaline phosphatase; TPT, triphenyltin; TBT, tributyltin; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; LXR, liver X receptor; RT-PCR, reverse transcription-polymerase chain reaction; FXR, farnesoid X receptor; ERR, estrogen-related receptor; ER, estrogen receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; VDR, vitamin D receptor; TIF2, transcriptional intermediary factor 2; hRXR, human retinoic acid receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; Dex, dexamethasone; Rosi, rosiglitazone; LG100268, 6-(1-(3,5,5,8,8-pentamethyl-5,6,7,8- tetrahydronaphthalen-2-yl)cyclopropyl)pyridine-3-carboxylic acid; TO-901317, *N*-(2,2,2-Trifluoroethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl)phenyl]benzenesulfonamide; GW501516, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl) phenoxy-acetic acid.

Here, we show that TPT and TBT are high-affinity ligands for RXR and PPARγ. Organotin compounds act as agonists of both RXRα and PPARγ in mammalian reporter gene assays and induce the expression of PPARγ target genes. PPARγ forms a heterodimer with RXR and binds to a defined DNA sequence in the promoter region of target genes (Mangelsdorf and Evans, 1995). PPARγ is activated by a variety of fatty acids and a class of synthetic antidiabetic agents, the thiazolidinediones (Lehmann et al., 1995). PPARγ serves as an essential regulator for adipocyte differentiation and lipid storage in mature adipocytes (Tontonoz et al., 1994). In light of these previous findings, we evaluated the effects of TPT and TBT on adipogenesis and found that organotins stimulate the differentiation of preadipocyte 3T3-L1 cells to adipocytes. Our data suggest that organotins exert their toxic effects through activation of the PPARγ/RXR signaling pathway.

Materials and Methods

Plasmids. The ligand-binding domains (LBDs) of the human nuclear receptors PPARα (codons 168–468; GenBank accession no. L02932), PPARγ1 (177–477; L40904), PPARδ (139–441; L07592), liver X receptor (LXR)α (167–447; U22662), and LXRβ (155–461; U07132) were amplified by RT-PCR from human liver mRNA as the template; the LBDs of human farnesoid X receptor (FXR) (193–472; U68233) and human estrogen-related receptor (ERR)γ (194–458; AF094518) were amplified similarly from human kidney mRNA and that of human ERRβ (195–434; AF094517) was amplified from human testis mRNA. The DNA sequences of the amplified fragments were confirmed by sequencing after subcloning into pGEX-4T (Amersham Biosciences Inc., Piscataway, NJ). The expression vectors for the human nuclear receptors estrogen receptor (ER)α/β, thyroid hormone receptor (TR)α, retinoic acid receptor (RAR)α/γ, RXRα/γ, vitamin D receptor (VDR), and human TIF2 were described previously (Kanayama et al., 2003). For expression in mammalian culture cells, the LBD of hRXRα was fused to the C-terminal end of the GAL4 DNA binding domain (amino acids 1–97) in the pBK-CMV expression vector (Stratagene, La Jolla, CA). The expression plasmid of (GAL4-DBD)-PPARγ (pM-mPPARγ1) and the luciferase reporter plasmid p4xUAS-tk-luc (Kamei et al., 2003) were kind gifts from Dr. Y. Kamei (National Institute of Health and Nutrition, Tokyo, Japan).

Chemical Reagents. Diethyl phthalate, triphenyltin chloride, nitrofen, 4-nonylphenol, octachlorostyrene, permethrin, triphenylmethane, and triphenylethylene were purchased from Kanto Chemical (Tokyo, Japan). Amitrole, 2,4-dichlorophenoxy acetic acid, 1,2-dibromo-3-chloropropane, γ-hexachlorocyclohexane (lindane), pentachlorophenol, dihexyl phthalate, di-*n*-pentyl phthalate, dipropyl phthalate, 2,4-dichlorophenol, 4-nitrotoluene, and bisphenol A were purchased from Tokyo Kasei (Tokyo, Japan). Chenodeoxycholic acid, 1α,25-dihydroxy cholecalciferol, lithocholic acid, all-*trans* retinoic acid, 9-*cis* retinoic acid, and 3,3′,5-triiodo-L-thyronine were purchased from Sigma-Aldrich (St. Louis, MO). 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2, rosiglitazone, and TO-901317 were purchased from Cayman Chemical (Ann Arbor, MI). GW501516 was purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan). The 40 chemicals tested and the abbreviations used for them are listed in Table 1.

Preparation of Proteins. The histidine-tagged fusion protein human TIF2 NID-BAP, in which the nuclear receptor interaction domain of TIF2, was ligated to the bacterial alkaline phosphatase (BAP), was expressed in *Escherichia coli* BL21 (DE3) cells and purified on Ni-nitrilotriacetic acid agarose resin (QIAGEN, Valencia, CA). Except for LXRα/β and FXR, the glutathione *S*-transferase fusion proteins were expressed in the *E. coli* BL21 (DE3) pLysS cells; LXRα/β and FXR were expressed in *E. coli* JM109 pRIL cells. The glutathione *S*-transferase fusion proteins were purified by using glutathione-Sepharose 4B (Amersham Biosciences Inc.).

CoA-BAP System. Detection of ligand-dependent interaction between nuclear receptors and TIF2 was carried out as described previously (Kanayama et al., 2003) but with slight modification. In brief, 2 μg of nuclear receptor protein diluted in 100 μl of carbonate buffer (100 mM $NaHCO_3$, pH 8.4) was incubated in the well of a 96-well polystyrene microtiter plate (MaxiSorp; Nalge Nunc International, Rochester, NY) at 4°C overnight. The plate was washed three times with 120 μl of buffer A (20 mM Tris-HCl, 100 mM KCl, 0.25 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol, and 0.05% Tween 20, pH 7.4), and then 100 μl of TIF2-BAP fusion protein (30 μg/ml) in buffer A was added to a well with the test chemical. After 1-h incubation at 4°C, the plate was washed three times with 120 μl of buffer B (50 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, and 0.10% Nonidet P-40, pH 7.2). The enzyme reaction was started by the addition of 100 μl of substrate solution (10 mM *p*-nitrophenyl phosphate in 100 mM Tris-HCl, pH 8.0). After incubation at 37°C for 30 to 90 min, the reaction was stopped by addition of 25 μl of 0.5 N NaOH. Finally, the absorbance at 405 nm was measured with a plate reader (MultiskanJX; Thermo Labsystems, Helsinki, Finland).

Cell Culture. Mouse 3T3-L1 (Dainippon Pharmaceutical, Osaka, Japan) and mouse NIH-3T3 (clone 5611, JCRB0615; Japanese Cancer Research Resources Bank, Osaka, Japan) fibroblasts were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% calf serum

TABLE 1
Suspected endocrine disruptors tested in this study

No.	Compound	Abbreviation	CAS No.
1	Diethyl phthalate	DEP	84-66-2
2	Dipropyl phthalate	DPrP	131-16-8
3	Di-*n*-butyl phthalate	DBP	84-74-2
4	Di-*n*-pentyl phthalate	DPP	131-18-0
5	Dihexyl phthalate	DHP	84-75-3
6	Diethylhexyl phthalate	DEHP	117-81-7
7	Dicyclohexyl phthalate	DCHP	84-61-7
8	Butyl benzyl phthalate	BBP	85-68-7
9	Diethylhexyl adipate	DEHA	103-23-1
10	4-Nonylphenol	4-NP	25154-53-3
11	*p*-Octylphenol	p-OP	1806-26-4
12	Bisphenol A	BPA	80-05-7
13	Triphenyltin	TPT	639-58-7
14	Tributyltin	TBT	1461-22-9
15	4-Nitrotoluene	4-NT	99-99-0
16	Benzophenone	BZP	119-61-9
17	Benzo[*a*]pyrene	B[*a*]P	50-32-8
18	Aldicarb		116-06-3
19	Vinclozolin		50471-44-8
20	Carbaryl	NAC	63-25-2
21	Methomyl		16752-77-5
22	Maneb		12427-38-2
23	Mancozeb		8018-01-7
24	Ziram		137-30-4
25	Methoxychlor	MXC	72-43-5
26	Hexachlorocyclohexane	γ-HCH	58-89-9
27	Permethrin		54645-53-1
28	2,4-D		94-75-7
29	2,4,5-T		93-76-5
30	Simazine	CAT	122-34-9
31	Alachlor		15972-60-8
32	PCP		87-86-5
33	Amitrole		61-82-5
34	Nitrofen	NIP	1836-75-5
35	Trifluralin		1582-09-8
36	1,2-dibromo-3-chloropropane	DBCP	96-12-8
37	Malathone		121-75-5
38	Kelthene		115-32-2
39	2,4-Dichlorophenol	DCP	120-83-2
40	Octachlorostyrene	OCS	29082-74-4

(MP Biomedicals, Aurora, OH). Mouse F9 embryonic carcinoma cells were maintained in 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS) (MP Biomedicals).

Transient Transfection Assays. One day before transfection, 1×10^5 cells were plated in a 35-mm dish containing phenol red-free minimum Eagle's medium (Nissui) supplemented with 10% charcoal/dextran-treated FBS. The cells were transfected by lipofection using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) with pBK-CMV-GAL4-hRXRα or pM-mPPARγ1 (300 ng/dish), p4xUAS-tk-luc (600 ng/dish), and RSV-βgal (100 ng/dish). Fresh medium with or without test chemical was added the day after transfection. After incubation for 24 h, cells were harvested and assayed for luciferase and β-galactosidase activity.

Adipocyte Differentiation Assays. Mouse 3T3-L1 preadipocyte cells were used for the differentiation experiments. The day after the cells reached confluence, the medium was replaced with DMEM containing 10% FBS, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX), and 1 μM dexamethasone (Dex). At the same time, the cells were treated with a test chemical (rosiglitazone, 9-*cis* retinoic acid, or an organotin compound). After 60 h, the medium was replaced with DMEM containing 10% FBS, 5 μg/ml insulin, and the test chemical. After 6 days, cells were fixed with 4% paraformalde-

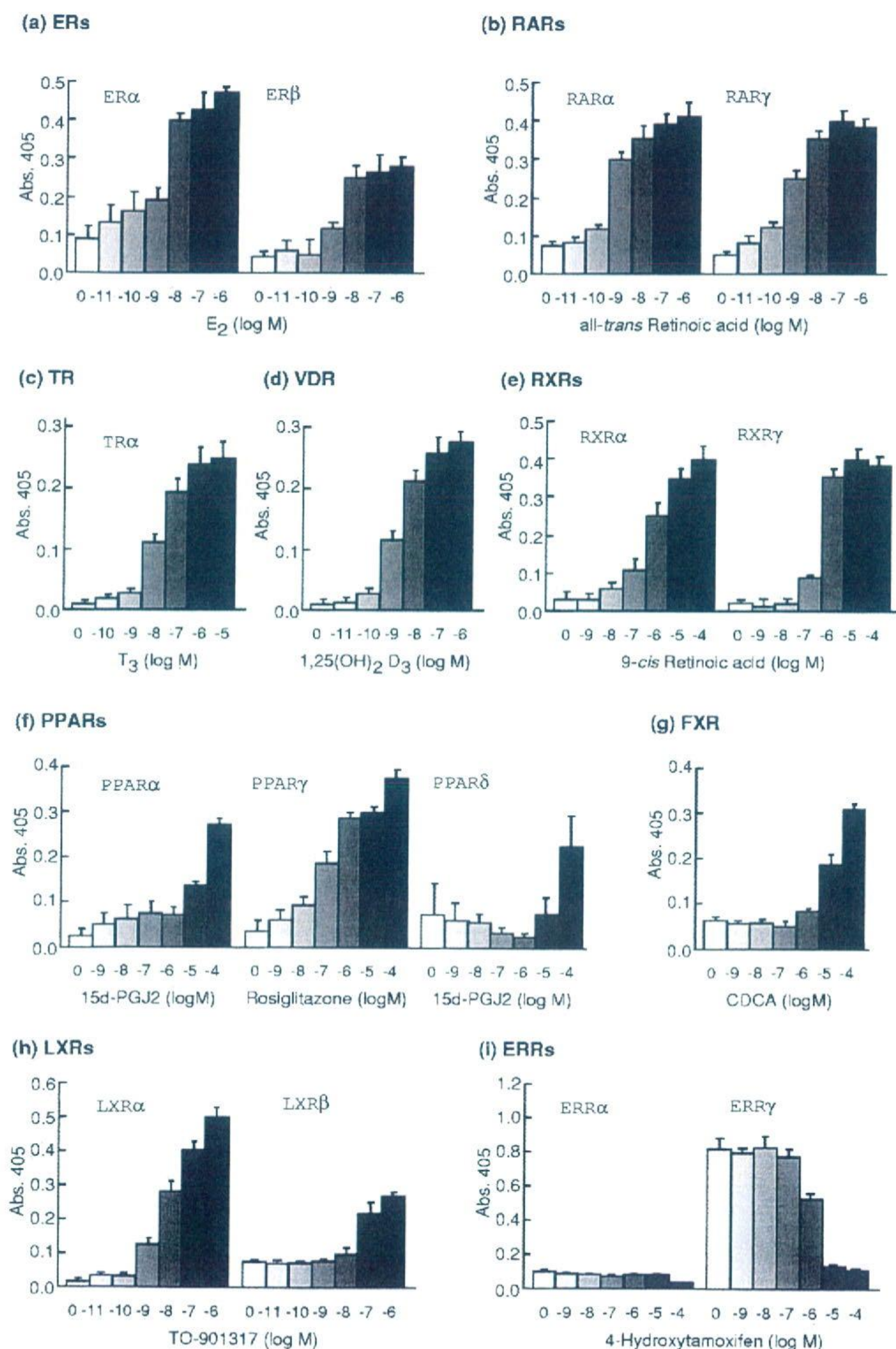


Fig. 1. Ligand-dependent interaction of nuclear receptor and TIF2 in vitro. Ligand-dependent interactions between nuclear receptors and TIF2-BAP were determined as relative alkaline phosphatase activity (vertical axis). The receptor-ligands pairs tested were ERα/β-17β-estradiol (E_2), RARα/γ-all-*trans* retinoic acid, TRα-3,5,3′-triiodo-L-thyronine (T_3), VDR-1α, 25-dihydroxy cholecalciferol [$1,25(OH)_2D_3$], RXRα/γ-9-*cis* retinoic acid, PPARα/δ-15-deoxy-$^{12,14}\Delta$-prostaglandin J_2 (PGJ2), PPARγ-rosiglitazone, LXRα/β-TO-901317, FXR-chenodeoxy cholic acid (CDCA), and ERRα/γ-4-hydroxytamoxifen. Data shown are means ± standard deviation of three independent experiments.

hyde and stained with 0.5% Oil Red O. The amount of triglyceride was determined by Triglyceride E Test (Wako Pure Chemicals).

RNA Isolation, Northern Blotting, and RT-PCR Analyses. The 3T3-L1 cells were grown in DMEM containing 10% calf serum. The day after the cells became confluent, they were treated with vehicle (dimethyl sulfoxide) only, rosiglitazone (Rosi), TPT, or TBT in DMEM containing 10% FBS and 5 μg/ml insulin. The cells were harvested at various times after treatment, and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). For Northern blot analyses, 25 μg of total RNA was electrophoresed through a 1% agarose gel containing 2% formaldehyde and then transferred to a Hibond-N$^+$ nylon membrane (Amersham Biosciences Inc.). The filter was hybridized with each probe, which was labeled with [α-^{32}P]dCTP by using a random labeling kit (TaKaRa, Shiga, Japan). For RT-PCR, cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan), and polymerase chain reaction was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA). The primers used for amplification of the aP2 gene (a marker for adipocyte differentiation) were 5′-AAAATGTGTGATGCCTTTGTGGG-3′ and 5′-TCATGCCCTTTCATAAACTCTTGTGG-3′.

Results

Application of CoA-BAP System to Endocrine Disruptors. Reproductive abnormalities in wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones. Because the nuclear receptors of intrinsic hormone systems are likely to be targets of industrial chemicals, information on their ability to bind these chemicals is valuable for environmental risk assessment. To determine whether suspected endocrine disruptors can bind to members of the nuclear receptor family, we constructed assay systems for human nuclear receptors, including ERα/β, RARα/γ, TRα, VDR, RXRα/γ, PPARα/γ/δ, FXR, LXRα/β, and ERRα/γ, on the basis of the previously described CoA-BAP system (Kanayama et al., 2003). The cognate ligand for each nuclear receptor enhanced alkaline phosphatase activity in a dose-dependent manner (Fig. 1). In the ERR systems, 4-hydroxy tamoxifen-dependent dissociations between ERR and coactivator were observed, as reported previously (Coward et al., 2001; Tremblay et al., 2001).

Using these systems, we evaluated 40 suspected endocrine disruptors (Table 1) recognized by various organizations (e.g., World Health Organization and Ministry of the Environment in Japan). The effects of the tested chemicals on the interaction between nuclear receptors and TIF2 (Fig. 2) suggest that several compounds possess agonistic activities for multiple receptors simultaneously. Butyl benzyl phthalate, hexachlorocyclohexane, maneb, mancozeb, and alkylphenols were weakly agonistic for multiple receptors, including ER. One intriguing finding was that the effect of TBT on RXRα was as strong as that of its endogenous ligand, 9-*cis* retinoic acid (Fig. 3), and the agonist effect of TPT on PPARγ was as strong as that of its well known ligand, Rosi (Fig. 3). The EC_{50} values of TBT on RXRα (7.4×10^{-8} M) and TPT on PPARγ (9.5×10^{-8} M) were almost the same as those of 9-*cis* retinoic acid (4.3×10^{-8} M) and Rosi (1.1×10^{-7} M), respectively. Because triphenylmethane and triphenylethylene were not agonistic for RXRα and PPARγ, the tin moiety was important for activity (Fig. 3).

Organotin Compounds Potentiated Transactivation by RXR and PPARγ. The observations that organotin compounds enhanced the protein-protein interaction between the coactivator TIF2 and RXRα or PPARγ suggested that these compounds activate transcription via these receptors. To confirm the results we obtained from the CoA-BAP system, we performed a reporter gene assay in mammalian culture cells using an expression vector for (GAL4-DBD)-RXRα or (GAL4-DBD)-PPARγ and a reporter plasmid containing the luciferase gene along with GAL4 upstream activating sequence. Both TPT and TBT induced the transactivation function of RXRα or PPARγ in a dose-dependent manner (Fig. 4). The effectiveness of these organotin compounds was comparable with that of known ligands. In addition, dibutyltin chloride, a TBT metabolite in vivo, also activated reporter activity in the PPARγ system (data not shown).

Induction and Promotion of Adipocyte Differentiation by Organotin Compounds in 3T3-L1 Cells. Recent studies indicate that PPARγ plays a central role in adipocyte gene expression and differentiation (Tontonoz et al., 1994). PPARγ is abundantly expressed in adipocytes, and its ligands induce the efficient conversion of fibroblastic cells to adipocytes, as measured by induction of adipocyte-specific genes and lipid accumulation (Lehmann et al., 1995). If or-

Fig. 2. Agonistic activities of suspected endocrine disruptors for various nuclear receptors. The effects of chemicals on the interaction between nuclear receptors and the coactivator TIF2 were assessed using the CoA-BAP system. The numbers in the far left column correspond to the chemicals listed in Table 1. The lowest effective concentrations of test chemicals were determined and compared with lowest effective concentration of cognate ligands shown in Fig. 1: red, ~1 to 10 times as much as cognate ligand; yellow, ~10 to 100; green, ~100 to 1000; gray, ~1000 to 10,000 times; and white, not detected. Triphenyltin (13) and tributyltin (14) showed strong activity on PPARγ and RXRα, respectively.

ganotin compounds can function as activators for PPARγ/RXR in vivo, these compounds probably induce adipocyte differentiation. To investigate this possibility, we treated 3T3-L1 cells with TPT or TBT in two types of differentiation medium, a complete differentiation medium that contained the inducers IBMX, Dex, insulin, and FBS and an incomplete differentiation medium that lacked IBMX and Dex. Although insulin is not always necessary for induction of differentiation, it efficiently enhances adipocyte development. Adipocyte differentiation was confirmed by staining with Oil Red O for lipid droplet accumulation. As expected, treatment of 3T3-L1 cells with either TPT or TBT in complete differentiation medium promoted adipocyte differentiation as well as did Rosi (Fig. 5, a–d). Even in incomplete differentiation medium, addition of organotin compounds induced adipocyte differentiation in contrast with the lack of induction after treatment with vehicle only (Fig. 5, e–h). Moreover, mRNA expression of the adipocyte differentiation marker aP2 was induced in a dose-dependent manner by addition of organotin compounds (Fig. 6a). PPARγ mRNA also was induced during the differentiation process (Fig. 6a), in agreement with the results of a previous study (Tontonoz et al., 1994). Induction of aP2 mRNA expression occurred late in adipogenesis (Fig. 6b), and organotin-treated cells demonstrated accumulation of triglyceride (Fig. 6c). Together, these data provide strong evidence that the organotin compounds TPT and TBT can function as inducers of adipocyte differentiation through PPARγ.

Discussion

Our study was designed to evaluate the effects of suspected endocrine disruptors on various nuclear receptors. The data show that several compounds have simultaneous effects on multiple nuclear receptors. In particular, organotin compounds (e.g., TBT and TPT) showed strong effects on RXR or PPARγ, at levels comparable with those of 9-*cis* retinoic acid, an endogenous RXR ligand, and rosiglitazone, a known agonist of PPARγ. In CoA-BAP systems, TBT showed strong effect on protein-protein interaction between RXRα and TIF2, but TPT showed slight effect (Fig. 3a). TPT showed strong effect on protein-protein interaction between PPARγ

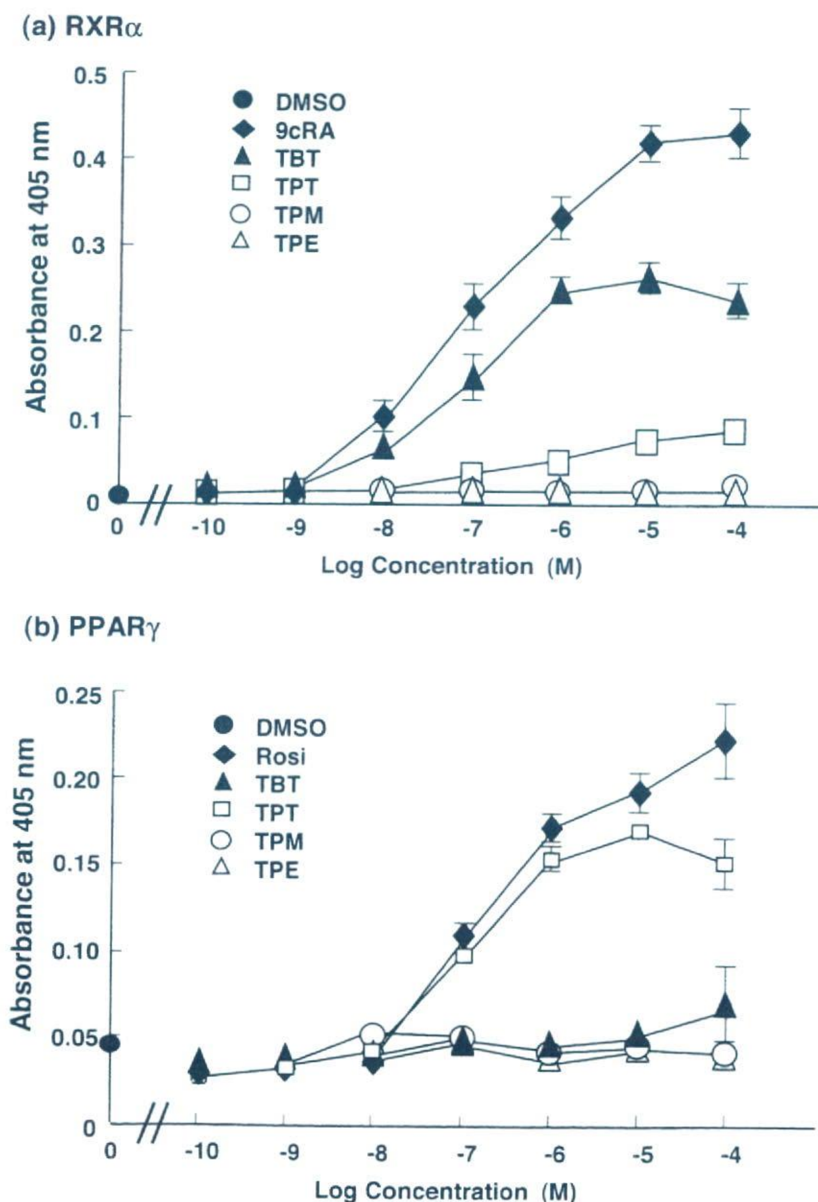
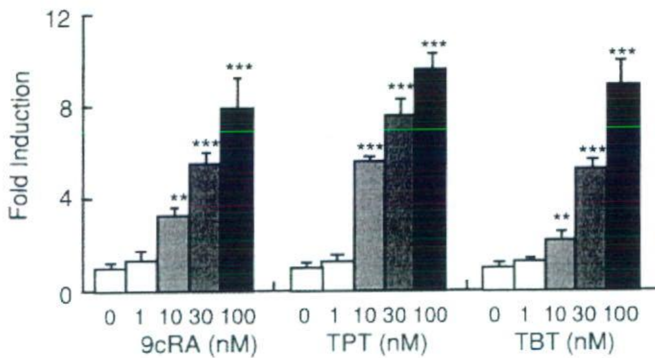


Fig. 3. Dose-response curves of the effects of organotin compounds on hRXRα and human PPARγ (hPPARγ) in the CoA-BAP system. A, TBT (▲) showed strong agonistic activity for hRXRα at as low a concentration as that of 9-*cis* retinoic acid (9cRA, ♦). B, TPT (□) showed strong agonistic activity to hPPARγ at as low a concentration as that of Rosi (♦). TPM (○) and TPE (△) did not show any agonistic activity. Activity of the vehicle control (dimethyl sulfoxide) only is shown by ●.

and TIF2, but TBT did not (Fig. 3b). On the contrary, when tested in the transactivation assay, both TBT and TPT activated not only RXRα but also PPARγ (Fig. 4). This discrepancy might reflect the diversity of coactivators. To date, many coactivators have been identified as nuclear receptor-interacting proteins. These coactivators are supposed to have cell- or tissue-specific functions in vivo (Smith and O'Malley, 2004). In addition, PPARγ reportedly changes its interaction partners depending on ligands (Kodera et al., 2000). We used only TIF2 in CoA-BAP system, whereas cells used for transactivation assays have many coactivators. The discrepancy of results from CoA-BAP systems and transactivation assays might be explained by this difference of coactivators. Because in vitro screening methods tend to produce false positive or false negative results like this, positive compounds should be further examined by other studies in a physiological context. Therefore, we examined the effects of organotin compounds on transcriptional regulation and adipogenesis, which is a famous physiological event related to PPARγ/RXR pathway.

Exposure of rats in utero to TBT induces a dramatic increase in the incidence of low-birth-weight fetuses because of maternal hypothyroidism (Adeeko et al., 2003). Furthermore, the RXR agonist bexarotene causes clinically significant hypothyroidism in patients with cutaneous T-cell lymphoma (Duvic et al., 2001), and experimental exposure of rats to LG100268 (a selective RXR agonist) induces the acute phase of hypothyroidism (Liu et al., 2002). The similarities between the toxicities of TBT and selective RXR agonists suggested to us that at least some of the toxic effects of organotin compounds are mediated by RXR.

Most of the toxic effects of organotin compounds on sexual development and reproductive function have been documented in mollusks (Matthiessen and Gibbs, 1998). In gastropods, TBT and TPT cause imposex (Morcillo and Porte, 1999), an irreversible syndrome in which male genital tracts (mainly a penis and a vas deferens) are imposed on female organisms (Smith, 1971). Although the physiological functions of organotin compounds have been studied extensively, the molecular target of organotin compounds had been unclear. To this end, we found that TPT and TBT were agonists for RXR and PPARγ. It has been thought that the sexual toxicity of organotin compounds results from increased androgen levels because of inhibition of the aromatase enzyme complex that catalyzes conversion of androgen to estrogen. This enzyme complex consists of microsomal CYP19 and the reduced form of the flavoprotein nicotinamide adenine dinucleotide phosphate reductase. TBT-induced imposex in neogastropods reportedly is mediated by inhibition of aromatase (Bettin et al., 1996), and TBT inhibits the catalytic activity of aromatase derived from transfected cells (Heidrich et al., 2001; Cooke, 2002). However, the effective concentrations of enzyme inhibition were relatively high (above 10^{-6} M). In this study, we found that TBT and TPT induced the transactivation function of RXRα and PPARγ at 10^{-8} M. It is reasonable that the effective concentration on gene expression was different from that on enzyme inhibition. In consistent with this, Nakanishi et al. (2004) demonstrated that 10^{-8} M TBT or TPT induced hCG or aromatase activity along with mRNA expression in placental cells (Nakanishi et al., 2002). In ovarian granulose cells, 20 ng/ml (about 6×10^{-8} M) TBT or TPT suppresses the $P450_{aroma}$ gene expression (Saitoh et al., 2001). We have to consider the toxicities of organotin compounds in distinguishing the low-dose effect from high-dose effect. Recently, we reported that RXR plays an important role in the development of gastropod imposex, by showing the cloning of RXR homolog from marine gastropod, binding of organotins to that receptor, and imposex induction by injection of RXR ligand 9-*cis* retinoic acid (Nishikawa et al., 2004). Gastropod imposex is known to be typically induced by very low concentrations of TBT and/or TPT (Bryan et al., 1986; Gibbs and Bryan, 1986; Horiguchi et al., 1997). Although it has been theorized that organotins increases androgen levels through inhibition of aromatase activity and/or a suppression of androgen excretion, the inhibitory concentration of organotins is not low enough for explaining imposex induction. The low-dose effects are likely to be mediated by receptors. However, the study of organotin effects in mammals is still important, because the compositions of nuclear receptor family members are very different between vertebrates and invertebrates (Escriva et al., 1997; Laudet, 1997). For example, there are no known homologs of steroid hormone receptors in the *Drosophila melanogaster* or *Caenorhabditis elegans* genomes, and the group members of TR, RAR, VDR, and PPAR seem to be late acquisitions dur-



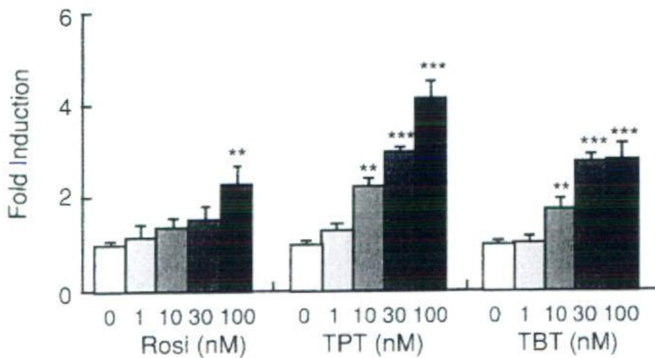


Fig. 4. Organotin compounds induce transcriptional activity through RXRα and PPARγ. Ligand-dependent transactivation of RXRα and PPARγ were detected as luciferase activity. a, F9 cells were cotransfected with a GAL4-DBD-hRXRα expression plasmid and a GAL4-responsive reporter plasmid. b, NIH-3T3 cells were cotransfected with a GAL4-DBD-mPPARγ1 expression plasmid and a GAL4-responsive reporter plasmid. The luciferase activities relative to the β-galactosidase activity are shown and represent the fold-stimulation compared with the activity of the vehicle-only control. Data shown are the means ± standard deviation of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ significantly different from vehicle controls.

ing the evolution of the superfamily. Therefore, we examined the effects of suspected endocrine disruptors on human nuclear receptor family members. As a result, PPARγ was identified as a new target molecule of organotin compounds in addition to RXR. This finding might introduce new insights in physiological functions of organotin compounds in mammals.

We were surprised to find that organotin compounds were high-affinity ligands for RXR and PPARγ. Until recently, it had been thought that among synthetic compounds, only hormone analogs could bind hormone receptors, because the relationships between hormones and their cognate receptors are very specific. However, some industrial chemicals do have unexpected effects on hormone receptors. Nuclear receptors are the likely targets, because their intrinsic ligands are fat-soluble, low-molecular-weight agents, as are the environmental pollutants. In fact, organotin compounds promote the adipocyte differentiation as agonists for PPARγ/

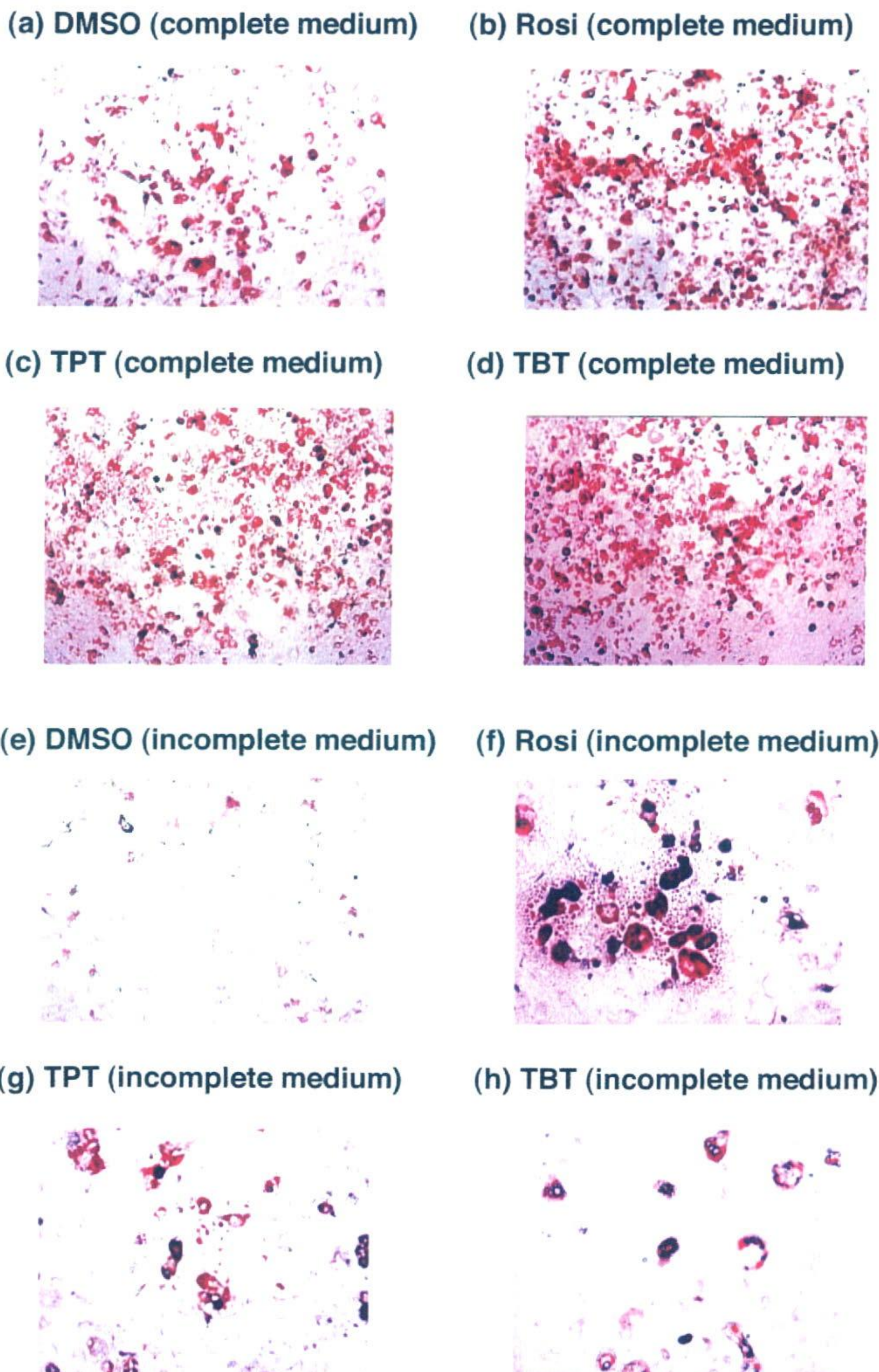
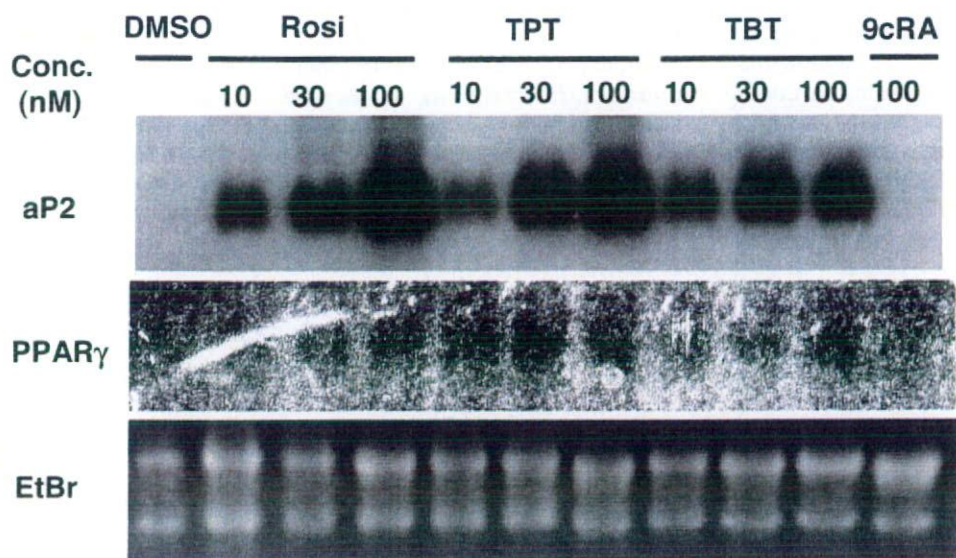
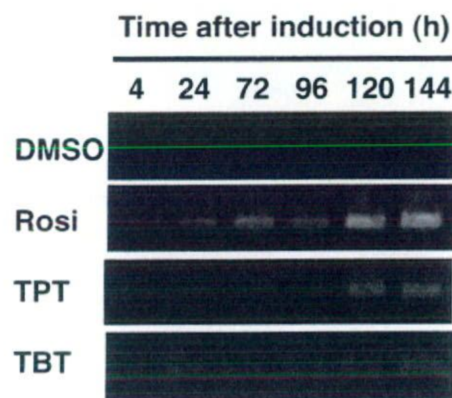


Fig. 5. Enhancement of lipid accumulation by organotin compounds. 3T3-L1 cells were maintained in DMEM containing 10% calf serum. One day after reaching confluence, the cells were treated for 60 h with vehicle only (a and e), 100 nM rosiglitazone (b and f), 100 nM TPT (c and g), or 100 nM TBT (d and h) in complete differentiation medium (a–d) or incomplete differentiation medium (e–h). The cells received fresh medium every 48 h. On the 10th day after induction of differentiation, the cells were fixed with paraformaldehyde and stained with Oil Red O.





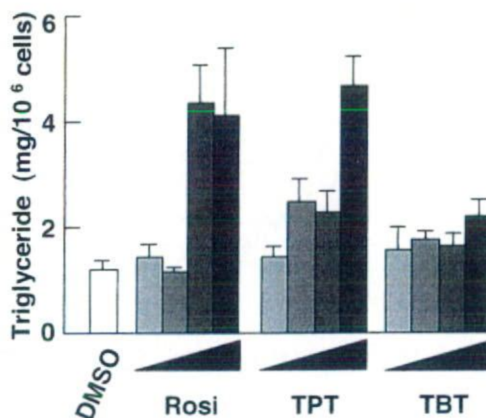


Fig. 6. Induction of adipocyte differentiation markers by organotin compounds. a, induction of adipocyte marker genes by organotin compounds in incomplete differentiation medium. 3T3-L1 cells were maintained in DMEM containing calf serum. One day after reaching confluence, the cells were treated with vehicle only, rosiglitazone (10–30 nM), TPT (10–30 nM), TBT (10–30 nM), or 9-*cis* retinoic acid (100 nM) in DMEM containing 10% FBS and 10 μg/ml insulin. Total RNA was isolated at 10 days after treatment, and mRNA expression of the aP2 and PPARγ genes was detected by Northern blot analysis. The ethidium bromide staining for ribosomal RNAs is shown as a control. b, time course of aP2 gene expression. 3T3-L1 cells were treated with vehicle only, rosiglitazone (100 nM), TPT (100 nM), or TBT (100 nM) in incomplete differentiation medium. The cells were harvested at the indicated time after treatment, and mRNA expression of the aP2 gene was analyzed by RT-PCR. c, lipid accumulation in differentiated 3T3-L1 cells. The cells were treated with 1, 10, 30, or 100 nM chemical. Ten days later, the amount of triglyceride was determined as described under *Materials and Methods.*

RXR. The ligands of PPARγ and RXR are expected for antidiabetic agents, but they have some side effects at the same time (Mukherjee et al., 1997; Yaki-Jarvinen, 2004). Although they may be good medicines when used under a doctor's control, wildlife are exposed to synthetic chemicals in uncontrolled manner. It is possible that TBT and TPT cause adverse health effects on the organisms by disturbing the endocrine process mediated by PPARγ/RXR.

Acknowledgments

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Address correspondence to: Dr. Jun-ichi Nishikawa, Department of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: nisikawa@phs.osaka-u.ac.jp

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Some organotin compounds enhance histone acetyltransferase activity

Shigehiro Osada[a,*], Jun-ichi Nishikawa[a], Tsuyoshi Nakanishi[b], Keiichi Tanaka[b], Tsutomu Nishihara[a]

[a] *Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka 565-0871, Japan*

[b] *Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka 565-0871, Japan*

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Abstract

Eukaryotic DNA is packaged into chromatin, whose basic subunit is the nucleosome, which consists of DNA and a core histone octamer. Histone acetylation is important for the regulation of gene expression and is catalyzed by histone acetyltransferase (HAT). We observed the effects of suspected endocrine-disrupting chemicals (EDCs) on HAT activity. We showed that some organotin compounds – tributyltin (TBT) and triphenyltin (TPT) – enhanced HAT activity of core histones in a dose-dependent way and other EDCs did not affect HAT activity. Organotin compounds have various influences on physical function including the hormone and immune systems, embryogenesis, and development. Dibutyltin and diphenyltin, metabolites of TBT and TPT, respectively, also promoted HAT activity, but monobutyltin, monophenyltin, and inorganic tin had no effect. Further, TBT and TPT enhanced HAT activity when nucleosomal histones were used as substrates. These data indicate that the organotin compounds have unique effects on HATs independent of their EDC activities and suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.
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Keywords: Histone acetyltransferase; Organotin; Tributyltin; Triphenyltin; Endocrine-disrupting chemical

1. Introduction

Nuclear eukaryotic DNA is packaged into chromatin, which has a major impact on levels of gene transcription. The basic unit of chromatin is the nucleosome core particle, which consists of 146 bp of DNA wrapped around a histone octamer. This octamer con-

* Corresponding author. Tel.: +81 6 6879 8242; fax: +81 6 6879 8244.
E-mail address: osada@phs.osaka-u.ac.jp (S. Osada).

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